NEONATAL MATERNAL SEPARATION IN MICE AS A PRE-CLINICAL MODEL FOR MALE CHRONIC PELVIC PAIN AND VOLUNTARY EXERCISE INTERVENTIONS

By

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Abstract

Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) is frequently co-diagnosed with other functional pain disorders, most significantly interstitial cystitis/painful bladder syndrome (IC/PBS), as well as mood disorders, particularly depression and/or anxiety. Patients suffering from functional pain disorders frequently report a history of early life stress, which has been associated with maladjusted stress response in adulthood. Basal function of the hypothalamic-pituitary-adrenal (HPA) axis, a major neuroendocrine stress response system, is programed during this critical period of development. Regular voluntary exercise has been shown to have a beneficial impact on depression and anxiety, as well as decreased perceptions of pain associated with HPA axis dysfunction in clinical and pre-clinical studies; however, to our knowledge, exercise has not been investigated as a potential intervention for comorbid urogenital pain disorders. This project reports neonatal maternal separation (NMS) induced mechanical perigenital allodynia, increased susceptibility to experimental colitis, altered micturition patterns, increased mast cell degranulation, and decreased HPA axis output. NMS did not, however, result in anxiety-like nor depression-like behaviors. Acute adult stress did significantly impact some of these measures, but did not enhance the effect of NMS. Lastly, we did see improvement of perigenital sensitivity following early and late exercise interventions, but did see more robust improvements in behavioral measurements after early exercise than late exercise. Late exercise, however, made a greater impact on central gene expression changes in limbic structures involved in HPA axis activity. Here, I have provided novel insight into the mechanisms potentially underlying significantly debilitating chronic pelvic pain disorders following early life stress, as well as evidence on the efficacy of therapeutic exercise, an easily translatable intervention, as a potential treatment strategy for these comorbid disorders. This work presents the first evidence of the impact of exercise on the effects of NMS on male mice, providing preclinical evidence for a treatment option for patients.

Dedication

This dissertation is dedicated to my encouraging and supportive family, Sabin and Myong Hee Fuentes, Anye Shin and Craig Park, Gabriella Fuentes, Peter Fuentes, and my steadfast partner Joshua Zornes.

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Table of Contents

List of Figures

Chapter I: Introduction

Visceral pain

The International Association for the Study of Pain (IASP) defines 'pain' as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" [1]. A particularly vexing type of pain for both patients and healthcare providers is chronic visceral or pelvic pain; pain that is localized to the lower abdomen and the pelvic and perineal regions. Chronic pelvic and urogenital pain is common and debilitating, and is the foremost complaint of patients suffering from functional bowel disorders (e.g., irritable bowel syndrome; IBS) and chronic pelvic pain disorders (e.g., interstitial cystitis, painful bladder syndrome; IC/PBS); moreover, this type of pain is idiopathic, meaning the etiologies of these painful disorders are unknown and are not associated with identifiable infectious, anatomical, metabolic, or other organic pathologies. The mechanisms of chronic visceral pain are poorly understood, in part due to its diffuse and poorly localized nature, often confused or overlapping between two visceral organs. Additionally, the diverse nature of visceral pain is compounded by multiple factors, including psychosocial stress, sexual dimorphism, and genetic and/or environmental predisposition. These multiple contributing factors make treatment and research efforts, especially the development and study of relevant animal models, challenging.

Despite its multifaceted nature, visceral hypersensitivity has been recognized to occur due to (1) sensitization of primary sensory afferents innervating the viscera (peripheral sensitization), (2) hyper-excitability of ascending spinal neurons receiving synaptic input from the viscera (central sensitization), and (3) dysregulation of descending pathways that modulate spinal nociceptive transmission [2]. For the purposes of this work, we will focus on the descending pathway and feedback loop of the hypothalamic-pituitary-adrenal (HPA) axis, as

well as its contribution to IBS, chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS), and IC/PBS.

Irritable bowel syndrome

Irritable bowel syndrome (IBS) is the most commonly diagnosed chronic pelvic pain disorder, as well as functional gastrointestinal disorder. IBS is characterized by chronic or recurrent abdominal pain or discomfort and altered bowel habits. IBS is estimated to impact approximately 20% of the US population and cost up to $30 billion in direct and indirect medical expenses annually [3, 4]. Like most functional pain disorders, IBS is diagnosed in twice as many women than men [5], and co-diagnosis with other chronic pelvic pain conditions is markedly high; up to 80% of IBS patients also present with symptoms of CP/CPPS, IC/PBS, and vulvodynia [6-11]. Additionally, up to 60% of IBS patients also suffer from anxiety and/or depression [12]. Moreover, a Norwegian study reported IBS patients with comorbid symptomology to have healthcare costs ten times that of patients with IBS alone [13].

A prominent symptom of IBS, and the main reason patients seek medical care, is visceral hypersensitivity (VH), which is defined by altered sensation of normal physiological stimuli (allodynia) and enhanced perception of mechanical stimulus to the abdominal region (hyperalgesia) [14]. The underlying mechanism contributing to VH is likely multifactorial and has been shown to involve inflammation, psychosocial stress, and peripheral and central sensitization of nociceptive input [14]. Symptom onset or exacerbation is often triggered by stress in patients suffering from IBS [12, 15-18]. A reported history of early life stress, such as premature birth, neglect, abuse, loss of a parent or parental discord, is a significant risk factor for developing IBS in adulthood [19-21]. This is thought to be due to perturbations in proper functioning of the HPA axis, particularly over activity, as it has been noted in sub-populations of IBS sufferers [21, 22]. A potential role for corticotropin-releasing factor (CRF; a key signaling hormone within the HPA axis) in mediating comorbidity between psychological and chronic pain

disorders has been investigated in both clinical and preclinical settings. Over-activity of central corticotropin-releasing factor receptor 1 (CRF_1) signaling has been proposed to contribute towards comorbid anxiety/depression in female diarrhea-predominant IBS patients [23]. However, antagonist inhibition of CRF_1 activity has had mixed success in patients depending on IBS sub-type, such has high-anxiety and generalized diarrhea-predominant IBS [24, 25]. These studies emphasize the heterogeneity of etiology underlying IBS, despite similar symptomology. Further understanding of IBS and comorbidities will be essential in designing appropriate, personalized treatment strategies.

Because of the significant impact of early life stress on IBS development, a frequently utilized research model is neonatal maternal separation (NMS) in rodents, particularly rats. NMS will be explored in greater detail later within this chapter. NMS-exposed rodents exhibit many colorectal sensitivity, functional, and neuroimmune abnormalities observed in human cases. Colorectal sensitivity is assessed by measuring abdominal muscle contraction, termed visceromotor response (VMR), during colorectal balloon distention (CRD). Rodents exposed to NMS demonstrate increased growth factor and cytokine expression, including nerve growth factor (NGF), interleukin 6 (IL-6), IL-1β, IL-2, IL-4, IL-10, and interferon (IFN)-γ [26-29], as well as infiltration of mast cells [30-32], in the distal colon, all of which can sensitize peripheral nociceptors and enhance visceral perception [26-29, 33]. Mast cell infiltration and hypertrophy of sensory innervation has also been reported in biopsies from patients with IBS [34-36].

Chronic prostatitis/chronic pelvic pain syndrome

After convening two international consensus conferences, the National Institutes of Health (NIH) established a new definition and a four-category classification system of prostatitis syndromes [37]. Table 1 outlines these categories and their defining characteristics. Categories I (acute bacterial) and II (chronic bacterial) prostatitis are well-defined and usually resolve after antimicrobial therapy, but only account for less than 10% of prostatitis cases [38]. Category IV,

Table 1: Classification of prostatitis syndromes [37]

Category	Name	Characteristics
I	Acute bacterial prostatitis	• Acute bacterial, urinary tract infection • Increased urinary frequency and dysuria • Systemic infection, bacteriuria, and pyuria
II	Chronic bacterial prostatitis	• Persistent and/or recurrent bacterial, UTI
III	Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS)	• Pelvic and urological pain, urinary complaints, and sexual dysfunction • Absence of other urological diseases or disorders
IIIa	Inflammatory CP/CPPS	• Leukocytes present in expressed prostatic secretions, post-prostate massage urine, or seminal fluid
IIIb	Noninflammatory CP/CPPS	• No evidence of inflammation in expressed secretions, post-prostate massage urine, or seminal fluid
IV	Asymptomatic inflammatory prostatitis	• No history of genitourinary tract pain complaints • Inflammatory infiltrates in prostate tissue or secretions/fluids found during evaluation for other genitourinary tract issues

asymptomatic prostatitis, is usually only discovered while patients are undergoing evaluation for prostate cancer, infertility, or other genitourinary tract issues. Category III chronic prostatitis (CP/CPPS) is further divided into inflammatory and noninflammatory subtypes based on the presence or absence of leukocytes in prostatic secretions. There is currently no single diagnostic test for CP/CPPS, but is rather diagnosed after ruling out other urogenital diseases or disorders. The NIH chronic prostatitis symptom index (NIH-CPSI) is a questionnaire that captures the three most significant domains of chronic prostatitis: pain (location, frequency, and severity), voiding (irritative/storage and obstructive/voiding symptoms), and quality of life (including impact). The NIH-CPSI is a valuable and reliable tool used in clinical practice and research settings [38, 39]. Patients with pain duration of less than 3 months, lower genitourinary tract cancer (e.g., prostate cancer), gastrointestinal disorders (e.g., inflammatory bowel disease), active urolithiasis or urinary tract infection, radiation of chemical cystitis, acute urethritis, acute epididymitis, acute orchitis, functionally significant urethral stricture disease, or neurological disorder affecting the bladder are excluded from a CP/CPPS diagnosis [40].

On average, it's estimated 8.2% men experience prostatitis symptoms annually in the United States, ranging between a prevalence rate of 2.2% to 9.7% [40] (9% of men in Canada [38]) and a life time prevalence near 14% [41]. Of the four clinically-defined categories of prostatitis, category III CP/CPPS is the most prevalent at a rate approximated to be 6-12% [42] and accounting for 90% of all chronic prostatitis cases [37, 43]. CP/CPPS is the most common urological diagnosis among men under the age of 50, as well as the third most common among those over 50 [44, 45], accounting for 2 million outpatient visits recorded annually in the United States [42, 46, 47]; approximately 8% of urology office visits in the US [48] and about 3% of male outpatient visits in Canada [38]. The estimated annual cost to treat prostatitis, due to increased outpatient visits and pharmacy expenses, is $84 million [44, 47]; annual patients' costs are approximated to be $4,400, twice that estimated for low back pain or rheumatoid

arthritis [47]. CP/CPPS is characterized by chronic, idiopathic pain in the lower abdomen, rectum, perineum, prostate, penis, and/or testicles with or without urinary symptoms [44, 45] and is diagnosed symptomatically due to the lack of associated pathology [49]. CP/CPPS has detrimental effects on quality of life, comparable to myocardial infarction, angina, Crohn's disease, and diabetes [45, 50].

Published rodent models of CP/CPPS require experimental infection; injection of exogenous antigens, androgens or irritants; or invasive surgery [46] and, as such, are largely undefined in their mechanism of CP/CPPS development. A spontaneous rat model of prostatitis has been reported; however, it requires 20-24 weeks [51, 52] or 40-52 weeks [53] before symptoms are observed. Possibly the most common rodent model is the experimental autoimmune prostatitis (EAP) model; complete Freund's adjuvant (CFA) is injected subcutaneously to produce abdominal/pelvic hypersensitivity. CFA is a solution of carrageenan in saline widely used to induce swelling and hypersensitivity in a variety of inflammatory models; however, studies utilizing the EAP model have resulted in varied degrees of prostatic inflammation depending on the species and strain used [43, 54-57]. Another rodent model initiates direct inflammation of the prostate by intraorgan injection of zymosan either once or several times [58, 59]. Proposed mouse models, however, have lacked translational potential due to unclear mechanisms of action, nonspecific results, high mortality, and/or lack of validation [46].

Interstitial cystitis/Painful bladder syndrome

Similar to CP/CPPS, IC/PBS is characterized by idiopathic pelviperineal pain and increased urinary urgency and frequency. IC/PBS is also diagnosed based on symptoms, particularly irritative voiding and referred lower urinary tract pain, after excluding other pathologies that mimic symptoms of IC/PBS (e.g., urinary tract infection). According to the Interstitial Cystitis Association (ICA), IC/PBS impacts 3 to 8 million women (3-6% of all women

in the US) and 1 to 4 million men in the United States [60]. Other sources have estimated as high as 11% of women and 5% of men in the US meet the high sensitivity definition of IC/PBS [61-63]. Patients' direct medical costs for treatment are estimated to be about $4000 annually [64], translating to roughly $20 to $40 billion a year spent in the US alone.

IC, before it became the more encompassing IC/PBS, only referred to observable findings of tissue damage (i.e., Hunner's ulcers, glomerulations, and overt histopathology) [65, 66]; however, studies have revealed the population to be more heterogeneous than the strict criteria used for research qualifications. IC/PBS, like many idiopathic pain disorders, is considered polysyndromic and patients may exhibit one of multiple subtypes differentiated by additional features [67]. For example, IC/PBS can be further divided into ulcerative and non-ulcerative subtypes. This is determined by the presence of Hunner's ulcers or glomerulations (submucosal hemorrhages) without ulcers on the bladder wall identified during cystoscopic examination; however, about 10% of patients show no signs of Hunner's ulcers or glomerulations [68]. The reliance on cystoscopic criteria may be one reason IC/PBS is considered to be underdiagnosed. Today, diagnoses require (1) pain/discomfort for a duration equal to or greater than 6 weeks, (2) pain/discomfort perceived to be related to the urinary bladder, (3) at least one additional urological symptom (urgency and/or frequency), and (4) these symptoms cannot be attributed to other known causes of bladder pain (e.g., infection or organic disease) [65-67, 69].

A variety of possible etiologies have been proposed over the years, and, when taken together, suggest a cyclical cascade of events involving neurogenic inflammation, hyper-responsive immune system, urothelial lining leakage/dysfunction, and chronic pain; however, the primary insult that initiates IC/PBS pathophysiology is unclear [70]. Though the prodome has not yet been identified, several urine biomarkers have been associated with IC/PBS, including IL-6 [71-74], histamine [73], and nerve growth factor (NGF) [75, 76].

Using rodent models, intravesicular irritant injection has been used to study the effect neonatal bladder irritation on bladder sensitivity and function. Rats subjected to zymosan irritation have displayed increased visceromotor response (VMR) during urinary bladder distention (UBD), increased micturition frequency with decreases in volume thresholds, and increased plasma extravasation and neuropeptide release after intravesicular mustard oil application [77, 78]. Other rodent models of stress-induced bladder hypersensitivity will be discussed later in this chapter.

Co-morbidities

As previously mentioned, the etiology of CP/CPPS is unknown and patients frequently present with symptoms of or are co-diagnosed with other functional somatic syndromes and/or mood disturbances (e.g., anxiety and depression) [8, 79-84]. CP/CPPS patients are frequently diagnosed with comorbid IBS (35%), chronic headache (36%), fibromyalgia (5%), and psychological disorders (48%) [80]. Similarly, approximately 40% of IC/PBS patients suffer comorbid chronic pelvic pain disorders [3, 8, 9, 85], and a significant proportion also suffer from fibromyalgia, allergies, and mood disorders [6, 79, 86-89]. Associations between anxiety and voiding disorders have also been reported [90] and interstitial cystitis (IC) patients with fibromyalgia, chronic fatigue syndrome, or rheumatoid arthritis had higher mean afternoon cortisol levels and increased pain during bladder filling than IC patients with no additional diagnoses [91]. Furthermore, there is an estimated 17% overlap between patients diagnosed with CP/CPPS and IC/PBS when considering only those patients that met the high sensitivity criteria for research qualification, meaning the diagnostic overlap of these syndromes may actually be greater [79]. As with CP/CPPS, current treatments for IC/PBS are largely unsatisfactory [80] and the high comorbidity between the two syndromes creates an even greater negative impact on quality of life, complicating already less-than-ideal treatment strategies [80, 89]. Not only is it difficult to treat the painful syndrome(s), it is also significantly

more difficult to treat depression symptoms in patients suffering from concomitant chronic pain [92].

The Interstitial Cystitis Association asserts the number of men affected by IC/PBS may be greater than currently estimated because it is often ‘mistaken’ for CP/CPPS; similarly, more recent studies have revealed CP/CPPS symptoms to be more widespread than previously thought, thus contributing to its under-diagnosis in the United States [63, 93]. Several investigators, however, have proposed IC/PBS and CP/CPPS to be different manifestations of the same underlying syndrome [93-98]. CP/CPPS and IC/PBS share multiple symptoms, specifically pelviperineal pain and urinary symptoms, and are frequently co-diagnosed [63, 65]. To further illustrate the overlap between these syndromes, cystoscopic findings of glomerulations in the bladder, a diagnostic symptom of IC/PBS, are also present for up to 70% of men with CP/CPPS, supporting the possibility that CP/CPPS and IC/PBS are one and the same [98].

Patients suffering from CP/CPPS often experience higher perceptions of stress, which can trigger or worsen ongoing symptoms [99, 100]; similarly, symptom onset and increased severity during periods of heightened stress are hallmarks of IC/PBS [11, 81]. Comorbid mood and pelvic pain disorders have been associated with disruption in proper functioning and limbic regulation of the hypothalamic-pituitary-adrenal (HPA) axis, which utilizes corticotropin-releasing factor (CRF) to regulate stress response and influence the perception of pain [21, 101-105].

Hypothalamic-pituitary-adrenal axis

The key system in the stress response is the hypothalamic-pituitary-adrenal (HPA) axis. In response to stress, the neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete corticotropin-releasing factor (CRF) and arginine vasopressin (AVP). This triggers the subsequent release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which in

turn signals the production of glucocorticoids (cortisol in humans, corticosterone in rodents) by the adrenal cortex. Similarly, family members urocortin (Ucn) 2 and Ucn3 are produced in stress-related brain regions, including the PVN [106, 107]; however, Ucn1 is largely expressed in Edinger-Westphal, superior lateral olive, and supraoptic nuclei [108, 109]. When the perceived stressor has subsided, feedback loops at various levels of the system (pituitary, hypothalamus, hippocampus, frontal cortex) trigger the shutdown of the HPA axis and a return to homeostasis through the activation of CRF receptors (CRF_1 and CRF_2) and the glucocorticoid receptors (glucocorticoid receptor (GR) and mineralocorticoid receptor (MR)) [110-113]. Activation of CRF_1 and CRF_2 work in opposition of one another, driving and dampening HPA output, respectively [114-117]. CRF binds CRF_1 with a 10-fold higher affinity than CRF_2, Ucn1 binds CRF_1 and CRF_2 with equal affinity, and Ucn2 and Ucn3 both preferentially bind CRF_2 [118]. The lower-affinity GR is richly expressed throughout the hippocampus and the prefrontal cortex, limbic structures that are implicated in the feedback regulation of the HPA axis [119, 120]. Through electrical and chemical stimulation, genetic manipulation, and lesion studies, these brain regions have been shown to be responsible for negative feedback inhibition of the stress response through GR binding, likely in parallel due to innervation of common subcortical targets [120, 121]. The high-affinity MR can be bound at low circulating levels of glucocorticoids, and is thus thought to be important in ambient glucocorticoid signaling [122].

These receptors are also prevalent within brain regions associated with affective, stress, and nociceptive circuitries. Studies have described central activation of CRF receptors mediating stress-related changes in GI function [123, 124]. Additionally, studies in children exposed to severe deprivation, neglect, or abuse report lower baseline levels of glucocorticoids [112, 125]. It has been hypothesized that this may be due to a downregulation of the HPA axis at the level of the pituitary in response to chronic drive of CRF from the hypothalamus [126], or target tissue hypersensitivity to glucocorticoids [127]. Limbic structures, including the frontal

Figure 1.1 Hypothalamic-pituitary-adrenal axis schematic

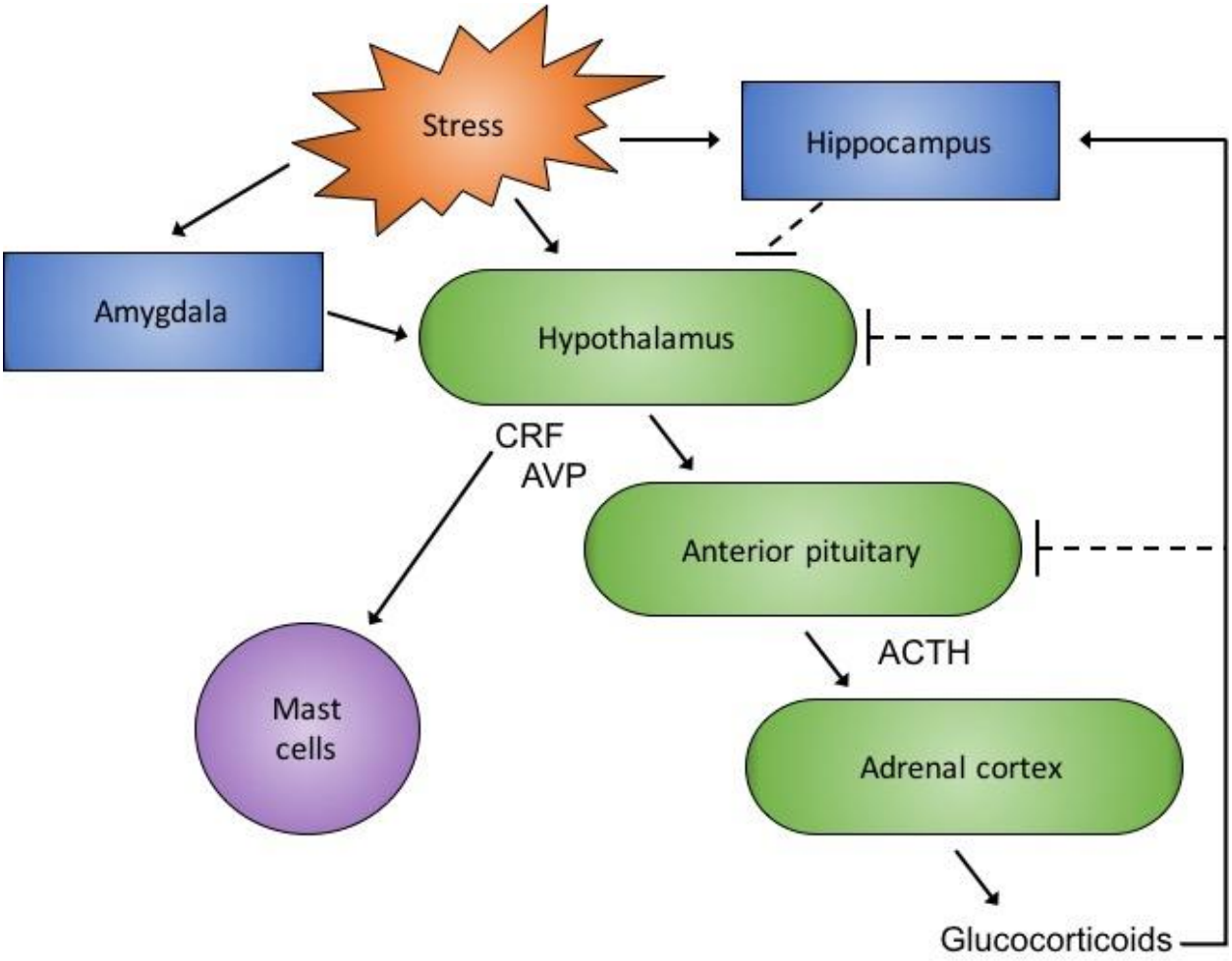

Figure 1.1 A schematic diagram of the HPA axis. When a stress perceived, neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete corticotropin-releasing factor (CRF) and arginine vasopressin (AVP). This triggers the subsequent release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which in turn signals the production of glucocorticoids (cortisol in humans, corticosterone in rodents) by the adrenal cortex. CRF can also be released in the periphery to act on mast cells and enteric neurons. Glucocorticoids act in negative feedback loops at various levels of the system (pituitary, hypothalamus, hippocampus, frontal cortex) to trigger the shutdown of the HPA axis and a return to homeostasis through the activation of CRF receptors (CRF_1 and CRF_2) and the glucocorticoid receptors (glucocorticoid receptor (GR) and mineralocorticoid receptor (MR)). In the hypothalamus and anterior pituitary, CRF and ACTH syntheses are halted, respectively. CRF and ACTH production are also arrested in the amygdala and hippocampus, limbic structures that respectively activate and inhibit the hypothalamus.

cortex, amygdala, and hippocampus, provide descending regulatory input to the HPA axis through interneurons that project to the PVN of the hypothalamus. Under normal conditions, the amygdala and hippocampus stimulate and inhibit CRF production/secretion from the PVN, respectively, working in opposition to one another to control the activation of the HPA axis [128, 129]. Stress exposure promotes CRF release in the central amygdala, a limbic structure involved in memory processing, decision-making, and emotional reactions [130]. Chronic glucocorticoid exposure increases expression of CRF mRNA [131, 132], suggesting a stress sensitization that may be involved in the development of stress-related pathologies [120].

Rodent models of have illustrated a potential role of the HPA axis in visceral organ sensitivity and function as well. Intrathecal administration of a CRF_2 antagonist prior to UBD was able to attenuate unpredictable footshock-induced urinary bladder hypersensitivity [133]. Treatment with CRF_1 antagonist reduced both bladder filling and micturition volumes that were initially increased due to intrathecal administration of CRF or Ucn2 [134]. Klausner *et al.*, however, observed the opposite effect. CRF administration decreased micturition volume in normal Wistar rats, and intrathecal administration of astressin, a non-selective CRF_1/CRF_2 antagonist, increased void volumes of high-anxiety Wistar-Kyoto rats [135]. In a recent study published from our lab, Pierce *et al.* showed NMS increased female VMR to vaginal balloon distention (VBD), as well as significant increases in IL-10 and NGF mRNA levels in the bladders of NMS-exposed female mice [136]. Additional work is underway to further investigate the effect of NMS on bladder sensitivity and function. Our lab has also shown NMS to also induce referred prostate sensitivity in male mice [137] (the impact of NMS in male mice will be further discussed in later chapters). Together, these studies suggest components of the HPA axis to be involved in bladder, vaginal, and referred prostatic sensitivity, but the mechanism within these genitourinary systems is still unclear.

Pelvic pain and urinary symptoms both contribute to the reduced quality of life and depressive mood experienced by CP/CPPS patients [100, 138]. Depression and catastrophizing (the tendency to employ a set of pain-associated cognitive appraisals referred to as ruminative, magnifying, and helpless when undergoing or anticipating pain [100, 139]), can also influence the HPA axis. Studies have shown that HPA axis dysregulation may lead to abnormal inflammatory responses, resulting in chronic inflammatory and pain conditions such as fibromyalgia, IC/PBS, and CP/CPPS [91, 100, 140, 141]. In fact, men with CP/CPPS have a greater waking cortisol slope compared to healthy, age-matched controls, suggesting a dysfunctional HPA axis [100, 140].

Mast cells

Mast cell activation and degranulation has been proposed as a possible underlying cause for both CP/CPPS and IC/PBS [93]. Mast cells are multifunctional immune cells that express high-affinity immunoglobulin E receptors and release potent inflammatory mediators including, but not limited to, leukotrienes, cytokines, serotonin, histamine, and proteases, such as tryptase [142]. Involved in the innate immune response, hematopoietic progenitors of mast cells develop in the bone marrow and are recruited to the peripheral tissues, primarily interfacing with the environment (e.g., the respiratory-, gastrointestinal-, and genitourinary tracts), where they take up residence and undergo maturation following a complex network of signaling and transcription factors [143, 144]. Mast cells can be activated to degranulate and release their stores of immune mediators by chemokines, such as monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1α (MIP-1α) [145, 146], as well as CRF and Ucn1 [147]. As mast cells are often found in close proximity to nerves and their contents are known to act on nociceptive fibers, it is strongly asserted that mast cells play a key role in peripheral sensitization in chronic pain.

The role of mast cells as important mediators of chronic pelvic pain has been appreciated more widely in recent years. Though studies have reported varied degrees of mast cell degranulation and activation, CP/CPPS biopsies were observed having altered granular structure [148] and decreased numbers of intact mast cells [149], suggesting mast cell activation without complete degranulation and increased rate of complete degranulation could occur in CP/CPPS. Biopsies from IC/PBS patients have similarly revealed increased mast cell infiltration [150-155] and close proximity to density populated substance P (SP)-immunoresponsive nerve fibers [156]. Concentrations of tryptase and carboxypeptidase A (CPA3), a marker of mast cell activation, have been found to be increased in urine samples from CP/CPPS patients [157]. Mast cell tryptase and nerve growth factor (NGF) levels have also been shown to be increased in expressed prostatic secretions from patients [43]. Mast cell tryptase has been demonstrated to be elevated in IC/PBS urines [158, 159]. Mast cell tryptase is a component of mast cell granules and is released during degranulation. Furthermore, elevated NGF levels in seminal plasma have been demonstrated to be directly correlated with pain severity [160, 161]. Elevated concentrations of NGF, histamine, and proinflammatory cytokines have been observed in IC/PBS patients' serum [162] and urine [74, 163-165].

Mast cells have been found in rat models of autoimmune (EAP) [54] and spontaneous prostatitis [166], as well as in a mouse model of NMS-induced visceral hypersensitivity studied in our lab [137]. EAP studies have reported increases in mast cell infiltration, activation, and degranulation after induction [43, 157, 167] ; moreover, the appearance of intact mast cells decreased with study progression [43] similar to increases of complete degranulation observed in CP/CPPS biopsies [149]. Other EAP model studies have reported increases in NGF expression, which has been known to enhance neuronal excitability and density in peripheral tissues [57, 166] Furthermore, mast cell-deficient $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice failed to develop pelvic mechanic allodynia 5 days following EAP induction, unlike wild type controls; and treatment with

the mast cell stabilizer disodium cromoglycate (cromolyn) or histamine receptor antagonists attenuated pain behaviors in EAP mice [43]. These studies strengthen association between mast cells and the development of CP/CPPS.

Neonatal maternal separation

Rodent models incorporating early life stress have demonstrated altered anxiety-like behaviors, increased colorectal sensitivity, and modified bowel function associated with disrupted functioning of the HPA axis. Neonatal maternal separation (NMS) in rodents has been used for several decades as a model of early life stress that significantly impacts the functioning of the HPA axis [168-171]. In rats, the neonatal period between postnatal day 1 (P1) through P14 is critical for neurological development. In humans, this period begins prenatally and lasts until age 5 [172]; the nervous system is exceptionally pliant to nurturing and adverse events during this critical window of development [173]. Maternal deprivation in rats lowered GR expression in the hippocampus and cortex, resulting in protracted responses to acute stress and deficient glucocorticoid feedback in rats [174]. Altered hypothalamic and limbic CRF receptor and GR expression has also been reported [174-177]. These animals also had increased corticosterone levels [178] and prolonged ACTH release following stressful events [171, 174, 175, 177]; induced depressive behaviors including heightened anxiety behaviors in an open field area [113, 168-170]; and increased visceromotor response (VMR) to colorectal distention [113, 179-181]. The hyperalgesia was exacerbated following exposure to water avoidance stress and was prevented by a preemptive administration of a selective CRF_1 antagonist [182], indicating that CRF may play a critical role in the development of visceral hypersensitivity. Altered functioning of the HPA axis likely drove the heightened sensitivity to acute stressors and anxiety-provoking situations in NMS rats [176, 183], as CRF mRNA and CRF_1 immunoreactivity were both elevated following NMS in PVN, amygdala, and locus coeruleus (LC) [177]. Moreover, NMS rats displayed significantly higher levels of c-Fos expression in the cingulate

cortex and superficial and deeper laminae of the spinal cord in response to colorectal distention than did naïve rats [184, 185], suggesting that early life stress likely induced functional changes in the central descending modulatory system that may have contributed to hyperalgesia [2].

Chronic stress compromises the hippocampal inhibition of the HPA axis through diminished receptor expression or ligand resistance, as in the case of GR [186], thereby reducing descending inhibition onto the PVN and in turn increasing CRF release and glucocorticoid production [128, 129]. NMS has been shown to alter expression of hippocampal CRF and glucocorticoid receptors, thus affecting limbic feedback. Hippocampal CRF_1 and CRF_2 were both found to be increased in NMS rats, both prior to and following acute stress exposure [176], whereas GR expression was significantly decreased [174, 175].

Exercise

Voluntary physical activity can favorably influence brain plasticity by facilitating neurogenerative, neuroadaptive, and neuroprotective processes [187]. Chronic voluntary physical activity also attenuates neural responses to stress in brain circuits responsible for regulating peripheral sympathetic activity; mitigating several harmful consequences of acute stress exposure [187]. In rodents, exercise has been shown to attenuate both anxiety- and depression-related behaviors in NMS rats by normalizing gene expression changes within limbic structures regulating HPA axis output [188, 189]. Chronic wheel running in rats prevents behavioral consequences of uncontrollable stress, including features of depression and anxiety [187, 190-192]. Free access to running wheels also normalized hippocampal GR and BDNF mRNA levels in NMS rats [188]. In a separate study, exercised NMS rats exhibited a significant decrease in depressive behavior compared to sedentary counterparts [189]. Clinically, exercise has been shown to reduce the effects of depression and anxiety, as well as decrease associated perceptions of pain [193, 194]. Exercise has also been shown to significantly improve symptom severity in patients with IBS [193, 195-199], fibromyalgia [200-202], and

depression and/or anxiety [203-205]. To our knowledge, exercise has not been investigated as a potential therapeutic intervention for NMS-induced visceral hypersensitivity.

Study significance

Experiencing adverse childhood events can permanently alter the functioning of the HPA axis, serving as a risk factor for developing functional pain disorders [101, 102, 206-212]. This is particularly worrisome in the U.S., as the child maltreatment rate has consistently risen over the past decade [213] and the birthrate is steadily rising only for women over the age of 40 [214-217]. Babies born to women of this age group are significantly more likely to be admitted to the neonatal intensive care unit (NICU) where they are exposed to numerous stressors, including periods of prolonged maternal separation [218]. At birth, a human child's glucocorticoid levels increase sharply in response to various stressors, such as physical exams and heel lances experienced by infants in the NICU, during a period when the HPA system is extremely labile [112]. Further understanding of the development and maintenance of chronic pelvic pain will better inform appropriate assessment and interventions, thus leading to improved physical and psychological function.

Due to the shared symptomology and comorbidity of functional pelvic pain disorders, it would be most advantageous to develop a reliable animal model depicting multiple conditions. This project repurposed a well-validated NMS model that results in colonic sensitivity indicative of altered HPA function [178, 179, 181, 184, 185]. Recent work from our laboratory has shown that NMS generates comorbid urogenital pain and anxiety behaviors in female mice [136]; and, in male mice of the same strain (C57BL/6), increases susceptibility to experimental colitis [219], induces referred prostate hypersensitivity, and increases mast cell degranulation in urogenital tissues [137]. We applied this model in male mice to more accurately replicate CP/CPPS and common comorbid disorders, particularly IC/PBS, depression, and anxiety.

Exercise has been shown to improve health outcomes for a multitude of diseases and has a beneficial impact on depression, with animal behavioral models corroborating [187]. Voluntary exercise has been shown to attenuate molecular and behavioral changes associated with a dysfunctional HPA axis and significantly improves symptom severity in patients with IBS [193, 195-199], fibromyalgia [200-202], and depression and/or anxiety [203-205]. Rodent studies have shown that voluntary exercise can normalize abnormal hippocampal gene expression resulting from NMS, thereby reinstating proper descending limbic control of the HPA axis [188]. To our knowledge, exercise has not been investigated as a potential therapeutic intervention for NMS-induced visceral hypersensitivity.

The objective of this project was to investigate if acute adult stress would exacerbate the effect of early life stress on comorbid chronic pelvic pain syndromes and psychological disturbances in male mice, to gain novel insight into the mechanisms underlying these comorbid disorders, and to understand whether voluntary exercise could prevent or reverse those effects. We intended to identify key modulators that could serve as potential pharmacological targets, as well as provide the first pre-clinical evidence on the efficacy of an exercised-based therapeutic intervention, an easily translatable and inexpensive intervention. This work will present the first evidence of the impact of exercise on the effects of NMS, providing preclinical evidence for a treatment option for patients. The central hypothesis of this project was that acute adult stress would intensify, while exercise would attenuate neonatal maternal separation (NMS)-induced long-term anxiety and dysfunction/hypersensitivity of the pelvic organs by restoring proper functioning of the HPA axis. We found that NMS exposure induced mechanical perigenital allodynia, increased susceptibility to experimental colitis, altered micturition patterns, increased mast cell degranulation, and decreased HPA axis output. NMS did not, however, result in anxiety-like nor depression-like behaviors. Water avoidance stress did significantly impact some of these measures, but did not enhance the effect of NMS. Lastly, we did see improvement of

perigenital sensitivity following both early and late exercise interventions, but did see more robust improvements in behavioral measurements after early exercise than late exercise. Late exercise, however, made a greater impact on central gene expression changes in limbic structures involved in HPA axis activity. Here, I have provided novel insight into the mechanisms potentially underlying significantly debilitating chronic pelvic pain disorders following early life stress. I also present evidence on the efficacy of therapeutic exercise, an easily translatable intervention, as a potential treatment strategy for these comorbid disorders. This work will present the first evidence of the impact of exercise on the effects of NMS, providing preclinical evidence for a treatment option for patients. Positive results from this study could potentially be applied to other functional pain disorders linked to early life stress, including fibromyalgia and migraine, thus broadening the impact of our findings. The benefits/impact of the work would be improving the quality of life of individuals suffering from these debilitating mood and pain disorders, as well as decreasing the overall annual expense of treatment for these comorbid disorders.

Clinical implications: Chronic pelvic pain disorders are not well understood in their pathology and are often co-diagnosed with other pain and/or mood disorders; thus, complicating treatment options. Here, I have provided novel insight into the mechanisms potentially underlying these debilitating disorders following early life stress. I also present evidence on the efficacy of therapeutic exercise, an easily translatable intervention, as a potential treatment strategy for these comorbid disorders.

Chapter II: Methods

This project involved two main arms: 1) to determine the impact of neonatal maternal separation and adult stress on male mice; and 2) to evaluate the therapeutic potential of an early-in-life or late-in-life voluntary exercise intervention.

Animals

All experiments were performed using male C57Bl/6 (Charles River, Wilmington, MA) born and housed in the Research Support Facility at the University of Kansas Medical Center. Mice were housed on a 12-hour light cycle from 600 to 1800 hours and received water and food *ad libitum*. All research performed conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals in accordance to the guidelines specified by the University of Kansas Medical Center Animal Care and Use Protocols.

Neonatal maternal separation

Pups were removed as whole litters from the home cage for 180 minutes (1100-1400 hours) daily from postnatal day 1 (P1) to P21, date of birth being P0. Litters were placed into clean glass beakers with bedding and enrichment from the home cage and held in an incubator at 33°C and 50% humidity for 21 consecutive days [137]. Naïve pups were unhandled outside of normal husbandry procedures. Naïve and mice that underwent neonatal maternal separation, termed NMS, were weaned on P22.

Trinitrobenzene sulfonic acid (TNBS) treatment

Naïve and NMS animals were anesthetized with inhaled isoflurane (4% induction, 2% maintenance) and secured on a platform that elevated the pelvic region approximately 5 cm above the working surface. A water-based lubricant (KY Jelly, Johnson & Johnson, New Brunswick, NJ) was liberally applied to the perianal region to avoid sensitization of surrounding somatic tissues. Mice received an intracolonic instillation of TNBS (0.1 mL of 50 mg/mL or 20

mg/mL in 50% EtOH) or saline (0.1 mL) using an oral feeding needle attached to a 50 μL Hamilton syringe. Mice remained in an elevated position for 5 minutes to prevent leakage. Mice were then allowed to recover from anesthesia and were returned to their home cages.

Water avoidance stress

Mice were exposed to water avoidance stress (WAS) for 1 hour of 1 day or 7 consecutive days within the first 6 hours of the light cycle. Individual mice were placed on round platforms (5 cm diameter) centrally affixed to the bottom of containers (36 cm length x 31 cm width x 27 cm depth) filled with room temperature tap water up to 1 cm below the top of the platform.

Electromyographic electrode implantation and colorectal distention

Electrode implantation was performed as previously described [220] to measure the visceromotor response (VMR) of naïve and NMS mice to colorectal balloon distention (CRD). Under inhaled isoflurane (4% induction, 2.5% maintenance) and aseptic conditions, the bare ends of two Teflon-coated stainless steel wires (3 mm; Grass Technologies, West Warwick, RI) were inserted into the right lateral abdominal musculature, secured via 5-0 prolene sutures, tunneled subcutaneously to a small incision made in the nape of the neck, and externalized for access during testing. Skin incisions were closed using 5-0 silk suture. Following recovery from anesthesia, mice were housed singly and allowed to recover for a minimum of 4 days before undergoing testing.

To facilitate balloon insertion and obtain proper restraint during CRD, mice were briefly sedated with inhaled isoflurane (4% induction, 2.5% maintenance) and a custom-made, catheterized polyethylene plastic balloon (1.5 cm length x 0.8 cm diameter) was inserted into the anus until the proximal end of the balloon was 0.5 cm from the anal verge (total balloon insertion, 2 cm) and secured the base of the tail with tape. The mouse was then placed into a

Broome-style rodent restraint (Kent Scientific, Torrington, CT), the free ends of the electrode wires were attached to a differential amplifier (Model 1700, A-M Systems, Sequim, WA), and the mice were allowed to recover from anesthesia for 30 minutes. The balloon was inflated with air from a compressed nitrogen tank equipped with a dual-stage low delivery pressure regulator (Matheson-Linweld, Kansas City, MO) and a separate pressure monitor (World Precision Instruments, Sarasota, FL) was used to manually regulate the pressure inside of the balloon. Each pressure (15, 30, 45, 60, and 75 mmHg) was applied three times for 20 seconds with a 4-min rest period in between. A custom-made distension control device (The University of Iowa Medical Instruments, Iowa City, IA) was used to control the gas flow through the system. Electromyographic (EMG) activity as amplified, filtered, and recorded on a personal computer with Spike 2 software (Cambridge Electronic Design, Cambridge, UK) for off-line analysis. The VMR was quantified by measuring the area under the curve for the entire distension period divided by the duration of the distension and expressed as a percent of baseline activity (10 seconds prior to CRD).

Voluntary wheel running

Exercised mice were housed in cages equipped with stainless steel running wheels (Mini Mitter; Bend, OK; or STARR Life Sciences Corp. Oakmont, PA). Quantification of wheel revolutions was monitored by Vital View Acquisition and Analysis Software (Harvard Apparatus, Holliston, MA) or STARR Life Sciences Vital View Activity Software version 1.1. Average running distance per week per animal or per pair was calculated using Microsoft Excel.

Bladder catheter implantation surgery for cystometry

Small rodent bladder catheter implantation was performed as previously described by Uvin et al. 2012 [221]. Under anesthesia of inhaled isoflurane (4%, 2% maintenance) and aseptic technique, a purse-string suture was placed in the dome of the bladder using a polypropylene 6-0 monofilament suture. A small cystostomy was performed using an 18 G

needle inside the purse string and a PE 50 polyethylene catheter with a small cuff at the end (sterilized and flushed with saline prior to implantation) was inserted through the hole. The purse-string was tightened and secured around the tube with a surgeon's knot. The catheter was checked for leaks then tunneled subcutaneously to a small incision made in the nape of the neck, and externalized for access during testing. The abdominal muscles were closed using polypropylene 6-0 monofilament sutures, leaving a passage for the catheter, and the skin incisions were closed using silk 5-0 sutures. Mice were immediately treated with 0.3 mg/kg buprenorphine, as well as twice daily for two days following surgery. Mice were singly housed and allowed to recover for 7 days before undergoing testing.

Cystometry testing

Testing was performed as previously described [221]. After 1 week of recovery from bladder catheter implantation surgery, mice were singly restrained within the Small Animal Cystometry Lab Station (Catamount Research and Development Inc., St. Albans, VT). The externalized catheter was connected to an infusion pump and pressure transducer. The animal was undisturbed for an acclimation period of 10 minutes, followed by a 20-minute acclimation period during which the bladder was infused with room temperature, sterile saline at a constant rate of 20 µL per minute. Infusion continued during a 30-minute testing period or until two discrete voiding events were observed. Voiding frequency and intervals, detrusor contraction frequency and amplitude, and intravesical pressure were displayed and recorded continuously using Cystometry Analysis Software (Catamount).

Behavioral analysis

All mice underwent a 30-minute acclimatization period within the testing room either the day of or one day prior to each behavioral test. For both thermal and mechanical hind paw sensitivity and mechanical perigenital sensitivity testing, the mice were acclimated to the

apparatus for 30 minutes prior to testing and the experimenter was blinded to the group status of the mice.

Open field testing

Activity in naïve and NMS mice was measured using a Force Plate Actimeter (BASi, San Diego, CA), which consists of a rigid, low-mass horizontal plate (44 cm x 44 cm) coupled to high sensitivity force transducers on each corner. A Plexiglass enclosure rests a few millimeters above the plate to create a transparent enclosure, all of which rests within a light and sound-attenuated box. Animals were individually placed into the middle of the testing area and allowed to move freely for 10 minutes. During this time, the software recorded the distance traveled and position of the mouse. The total distance traveled and percent of time spent in the perimeter (outermost 8.25 cm; increased time spent in the perimeter is indicative of anxiety [222]) was calculated and binned and the data from the second 5 minute bin is reported here.

Thermal analgesiometer testing

Naïve and NMS mice were placed in individual clear plastic chambers (11 x 5 x 3.5 cm) on the 30°C heated glass surface of a thermal analgesiometer (UARDG; Department of Anesthesiology, University of California San Diego, La Jolla, CA). A high intensity light (4.25 Amperes) was directed at the plantar surface of the hind paw and the latency to withdrawal from the stimulus was automatically recorded within 0.01 second. Alternating hind paws were tested for a total of three times per side with a minimum of 5 minutes between applications. The stimulus terminated automatically at 20 seconds to avoid tissue damage. Individual responses were averaged and group means were determined as previously described [223].

Mechanical sensitivity monofilament testing

Hind paw and perigenital sensitivity were assessed as previously described, [219] and [137] respectively. Mice were first acclimated to the sound proof testing room for 30 minutes

prior to testing. Naïve and NMS mice were placed into individual clear plastic chambers (11 x 5 x 3.5 cm) on a wire mesh screen elevated 55 cm above a table. The up-down method was performed to test mechanical sensitivity using a standard set of Semmes-Weinstein monofilaments (1.65, 2.36, 2.83, 3.22, 3.61, 4.08, 4.31, 4.74 g; Stoelting, Wood Dale, IL) [224, 225]. Beginning with the 3.22 g monofilament, mice received a single application to either the plantar surface of the right hind paw or scrotum. A negative response was followed by the next larger filament and a positive response (considered a brisk jerk or jump or licking the affected area) was followed by the next smaller filament. The experimenter continued to move up or down the series, depending on the previously elicited response, for an additional four applications after the first positive response was observed for a minimum of five or a maximum of nine total monofilament applications. The value in log units of the final monofilament applied in the trial series was used to calculate 50% g threshold for each mouse and group means were determined as previously described [224].

Sucrose preference testing

Two liquid preference paradigms were employed. When investigating the impact of NMS and/or WAS exposure on sucrose preference, mice were individually housed in and acclimated to BioDAQ Liquid Choice Unplugged Allentown cages (Biological Data Acquisition, New Brunswick, NJ) equipped with two Polysulfone BioDAQ drinking bottles. Over a 48-hour acclimation period, both bottles were with standard drinking water. Following this period, one bottle was replaced with 1% sucrose diluted in drinking water. Fluid volumes were recorded every 24 hours for 4 days to assess baseline sucrose preference prior to WAS exposure. After 1 hour WAS exposure, mice were returned to two-choice liquid preference cages and liquid consumption was recorded every 24 hours until 8d post-WAS. Additionally, from the beginning from the acclimation period to the end of the 8d post-WAS, positions of the bottles were swapped to control for possible side preference.

When investigating the impact of NMS and/or exercise on sucrose preference, mice were individually housed in and acclimated to the liquid choice cages for 24 hours. Following the acclimatization period, one bottle was filled with 1% sucrose solution. Fluid volume levels were recorded at the beginning and the end of a 24-hour testing period. For both paradigms, the total volume and percentage of 1% sucrose was calculated.

Elevated plus-maze testing

Anxiety-like behaviors were measured in mice as previously described by Walf and Frye [226]. Mice were placed in the center of an elevated (60 cm above the floor) plus-maze consisting of two open and two closed arms opposite each other in a plus-shaped formation. Testing conditions were kept as consistent as possible. All mice were tested within the first seven hours of the light cycle and placed into the center of the maze facing the same open arm. Mice were allowed to freely explore the maze for a testing period of 5 minutes while a digital camera captured the activity from above. The number of entries into and the time spent in open and closed arms were recorded at the time of testing in the event a video recording was unusable. Blinded scorers reviewed the recorded test sessions and were able to record the number of entries and time spent in each arm more accurately.

Micturition analysis

Surfaces were cleaned with 70% ethanol and wiped dry. Naïve and NMS mice were acclimated to the testing room in their home cages to the testing room for 30 minutes prior to micturition pattern data collection. Mice were singly confined to a sheet of filter paper (11 x 5 x 3.5 cm) for 1 hour using an inverted Micro-Isolator cage bottom (Lab Products, Seaford, DE). At the end of the testing period, the filter paper was left to dry while mice were returned to home cages and the animal facility. Once the filter papers were dry, fecal pellets were counted (data not shown) and urine spots were visualized using UV light. The total area (cm^2) and number of urine spots were recorded.

In vitro assays

Myeloperoxidase assay

Mice were overdosed with inhaled isoflurane (>5%) and the distal 1.5 cm of colon was dissected and the fecal matter, excess blood vessels, and mesentery were removed. The tissue was weighed, added to a beaker containing 1 ml 0.5% hexadecyltrumethylammonium bromide (HTAB; Sigma), and finely minced using spring scissors. The solution was transferred to a 15 ml centrifuge tube, along with another 2 ml HTAB, and sonicated for 10 s before being homogenized for 30 s. Another 2 ml HTAB was added and the tube was placed on dry ice until all samples were similarly processed. The samples underwent three freeze-thaw cycles, were centrifuged twice, and loaded in triplicate, along with MPO standards (Calbiochem, San Diego, CA), onto a 96-well plate. The standards and samples were reacted with O-dianisidine dihydrochloride (Sigma) and read on a plate reader at 460 nm every 20 s for 15 min. The slope for each standard reading was calculated and plotted and the slope of those values was used to calculate the units of MPO activity/tissue weight for each sample.

Acidified toluidine blue mast cell staining

Mast cells were stained and quantified as previously described [137]. Mice were overdosed with inhaled isoflurane (>5%) and intracardially perfused with ice cold 4% paraformaldehyde. Urinary bladders and prostates were removed, post-fixed in paraformaldehyde at room temperature for 1 hour, cryoprotected in 30% sucrose in 1x PBS at 4°C overnight, and frozen in a heptane bath on dry ice. Tissue was transversely cut into 10 µm-thick (bladder) and 7 µm-thick (prostate) cryosections using a cryostat held at -22°C. Cryosections were then stained with acidified toluidine blue to visualize mast cells. The percentage of degranulated/total mast cells was calculated in 8 separate, non-serial sections spanning the length of each tissue.

mRNA extraction and qRT-PCR

Mice were overdosed with inhaled isoflurane (>5%). The 1.5 cm distal segment of the colon was removed, bisected longitudinally (to facilitate both mRNA and protein [see below] analysis), snap frozen in liquid nitrogen, and stored at -80°C. Total RNA was isolated using Trizol reagent (Ambion, Austin, TX) and RNeasy Mini Kit (Qiagen, Valencia, CA). The concentration and purity were determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and cDNA was synthesized from total RNA (0.63 µg) using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative RT-PCR was performed using SsoAdvanced SYBR Green Supermix (Bio-Rad) and a Bio-Rad iCycler IQ real time PCR system with indicated 20µM primers (Integrated DNA Technologies, Coralville, IA) listed in Table 2.1. GAPDH was used as a housekeeping gene for brain tissues and β-actin was used as a housekeeping gene for bladder and prostate.

Samples were run in triplicate and negative control reactions were run with each amplification series. To reduce variability among efficiency due to fluctuations in baseline fluorescence, the raw (i.e. non-baseline corrected) PCR data was imported to the LinRegPCR software (version 2012.3) [227-229] and PCR efficiency values were derived for each individual sample by fitting a regression line to a subset of data points within the sample's log-linear phase. Threshold cycle (Ct) values were subtracted from that of the selected housekeeping gene and the percentage of fold change over naïve controls was calculated using the Pfaffl method [230].

Table 2.1 Primers used for real-time PCR analysis

Gene	Forward (5’ – 3’)	Reverse (3’ – 5’)
IL-6	CTGCCAGAGACTTCCATCCAGTT	GAAGTAGGGAAGGCCGTGG
IL-10	GCTGGACAACATACTGCTAACC	ATTTCCGATAAGGCTTGGCAA
Artemin	GGCCAACCCTAGCTGTTCT	TGGGTCCAGGGAAGCTT
SCF	CCCTGAAGACTCGGGCCTA	CAATTACAAGCGAAATGAGAGCC
MCP-1	AGGTCCCTATGGTGCCAATGT	CGGCAGGATTTTGAGGTCCA
NGF	ACACTCTGATCACTGCGTTTTTG	CCTTCTGGGACATTGCTATCTGT
CRF	CCTCAGCCGGTTCTGATCC	GCGGAGGAAGTATTCTTCACCC
Ucn2	ACCCGTGTCATACTCTCCCTG	CAGCCTTGTAACGAGCCTG
CRF_1	CCCTGCCTTTTTCTACGGTGT	TTCCCGGTAGCCATTGTTTGT
CRF_2	CCTGTGGACACTTTTGGAGCA	TGTTGCAGTAGGTGTAGGGAC
GR	GACTCCAAAGAATCCTTAGCTCC	CTCCACCCCTCAGGGTTTTAT
MR	GAAAGGCGCTGGAGTCAAGT	CCATGTAGCTGTTCTCATTGGT
BDNF	CAGGTTCGAGAGGTCTGACGA	CGCGTCCTTATGGTTTTCTTCG
β-actin	AGTGTGACGTTGACATCCGTA	GCCAGAGCAGTAATCTCCTTCT
GAPDH	ATGTGTCCGTCGTGGATCTGA	ATGCCTGCTTCACCACCTTCTT

Western blot

Total protein was isolated from approximately 50 mg of snap-frozen colon, bladder, and prostate tissue using Cell Extraction Buffer (Invitrogen, Grand Island, NY) containing Halt protease and phosphatase inhibitors (ThermoFisher Scientific, Waltham, MA) and Na_3VO_4 (Sigma, St. Louis, MO). Protein concentrations were determined using a D_C protein assay (ThermoFisher). Samples were reduced by heating to 95°C for 5 minutes in the presence of 2-mercaptoethanol, subjected to SDS-PAGE (Criterion 4 – 12% Bis-Tris gels; Bio-Rad, Hercules, CA), and transferred to Nitrocellulose transfer membrane (Whatman GmbH, Dassel, Germany) by Criterion Blotter wet transfer (Bio-Rad). The membranes were blocked for 1 hour at room temperature in 5% milk in Tris-buffered saline with Tween-20 (TBST) and incubated overnight at 4°C with antisera to CRF_1, CRF_2, and GAPDH diluted in 5% milk in TBST. Membranes were then washed with TBST and incubated for 1 hour with anti-rabbit secondary. Dilution ratios of primary antibodies are reported in Table 2.2. Densitometry was performed using Quantity One 4.6.9 software (Bio-Rad).

Table 2.2 Antibodies and dilution ratios used for Western blot analysis

Antibody	1d WAS colon	8d WAS bladder	8d WAS prostate
CRF_1 (Millipore, Billerica, MA)	1:250	1:500	1:333
CRF_2 (Millipore)	1:1000	1:800	1:1000
GAPDH (Cell Signaling Technology, Danvers, MA)	1:2000	1:2000	1:2000
Anti-rabbit secondary (Cell Signaling)	1:2000	1:10,000	1:10,000

Serum corticosterone ELISA

Trunk blood was collected at the time of sacrifice during the early half of the light-cycle (0800-1100 hours), allowed to clot for 1 hour on ice, and centrifuged at 10,000 rpm for 10 minutes. Serum (clear supernatant) was collected and stored at -20°C until analysis. Serum corticosterone (CORT) was quantified using ELISA kit according to manufacturer's instructions (ALPCO, Salem, NH).

Statistics

Calculations were made using Microsoft Excel and statistical analysis was performed using Student's *t*-test or two-way (with or without repeated measures) analysis of variance (ANOVA) followed by Bonferroni's or Fisher's LSD posttests, and correlations were analyzed using Pearson's correlation (IBM SPSS Statistics, IBM Corporation, Armonk, NY; GraphPad Prism 6, GraphPad Software, La Jolla, CA), as denoted in the manuscript. All data are expressed as mean ± SEM. A p value of less than 0.05 was considered significant.

Chapter III: Results: Characterizing NMS in male mice (colon)

Experimental design

Naïve mice and mice subjected to neonatal maternal separation (NMS) from postnatal day 1 (P1) though either P14 or P21 were assessed as adults for open field behavior, hind paw sensitivity, and visceromotor response (VMR) to colorectal distension (CRD). VMR was also measured before and after treatment with intracolonic trinitrobenzene sulfonic acid (TNBS; 2 mg or 5 mg) or exposure to acute (1 day) or chronic (7 days) water avoidance stress (WAS). Myeloperoxidase (MPO) activity, proinflammatory gene and corticotropin-releasing factor (CRF) receptor expression were measured in distal colon. Table 3.1 describes the ages of the mice at experimental time points.

NMS increased hind paw sensitivity, but not baseline colorectal sensitivity or anxiety measures

Male mice underwent NMS or remained unhandled (naïve) for the first 21 days of life and were assessed as adults for susceptibility to colorectal inflammation and acute or chronic exposure to WAS (Table 3.1). At baseline, naïve and NMS mice were not remarkably different in regards to colorectal sensitivity, measured as the VMR during CRD (Figure 3.1A). Open field exploratory behavior and hind paw mechanical and thermal sensitivity were also measured in naïve and NMS mice at baseline and following CRD. The percent of time spent in the perimeter of the open field was not significantly different between naïve and NMS mice at baseline; however, exposure to CRD significantly increased overall time spent in the perimeter, specifically in NMS mice (Figure 3.1B). The withdrawal threshold to application of Semmes-Weinstein monofilaments to the plantar surface of the hind paw was significantly reduced in NMS mice, particularly post-CRD (Figure 3.1C). Thermal withdrawal latency to a radiant heat source on the plantar surface of the hind paw was significantly shorter in NMS mice at baseline, but not following CRD (Figure 3.1D).

Table 3.1 Age of mice at experimental time points

	Baseline	Insult	Post-insult measurements
Colorectal distension			
No insult (n=10)	13		
Saline (n=6)	8	9	10
TNBS 2 mg (n=6)	10	11	12
TNBS 5 mg (n=6)	8	9	10
1d WAS (n=6)	10	10	11
7d WAS (n=6)	10	10-11	12
Open Field			
CRD (n=8)	6	13	17
Hind paw Sensitivity			
CRD (n=8)	7	13	19

Naïve and NMS mice underwent behavioral or physiological testing at the above noted ages (in weeks) prior to and/or following introduction of an insult, including colorectal distension (CRD), intracolonic trinitrobenzene sulfonic acid (TNBS), or acute (1d) or chronic (7d) exposure to water avoidance stress (WAS). Repeated tests were performed on the same mice for all CRD experiments involving post-insult measurements. Open field and hind paw sensitivity tests were performed on separate groups of mice either at baseline or following CRD.

Figure 3.1 Colorectal, mechanical, and thermal sensitivity and behavior after colorectal distension

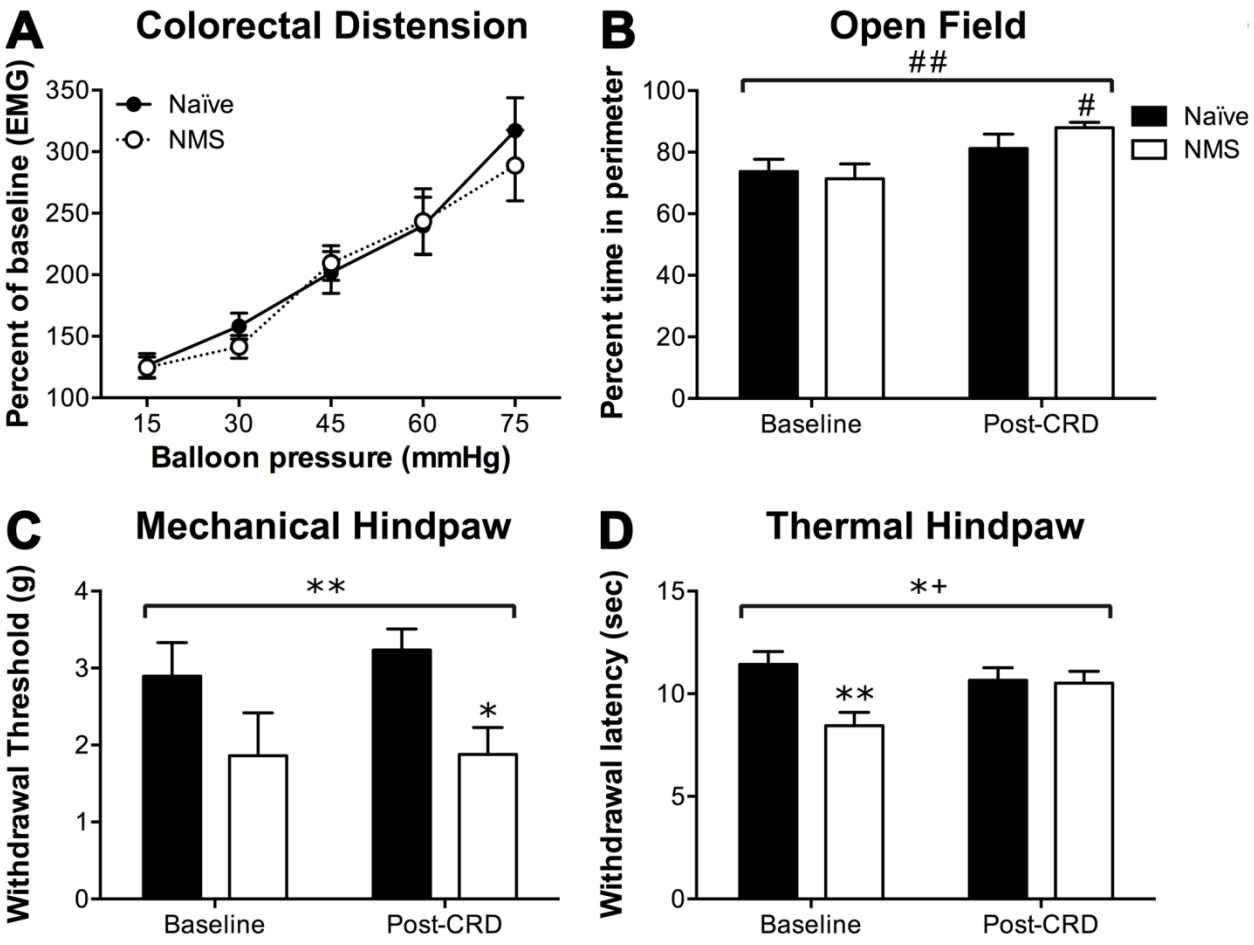

Figure 3.1 Colorectal, mechanical, and thermal sensitivity and behavior after colorectal distension. The visceromotor response (VMR) during colorectal distension (CRD) was measured, as well as open field activity and mechanical and thermal hind paw sensitivity prior to and following CRD, in male mice that did and did not (naïve) undergo neonatal maternal separation (NMS). **A)** No difference in VMR, measured as the electromyographic (EMG) activity of the abdominal muscles during CRD, was observed between naïve and NMS mice. **B)** At baseline, naïve and NMS mice spent a similar percent of time in the perimeter of an open field; however, following exposure to CRD, there was an overall significant increase in the percent of time spent in the perimeter, specifically in NMS mice. **C)** Mechanical hind paw withdrawal thresholds were significantly reduced in NMS mice across both measurements, particularly post-CRD. **D)** Thermal hind paw withdrawal latencies were significantly shorter in NMS mice at baseline, compared to naïve, but not post-CRD. An overall effect of NMS and an NMS/CRD interaction was observed across both measurements. Brackets indicate a significant effect of NMS (*, ** $p < 0.05, 0.01$), CRD (## $p < 0.01$), or an NMS/CRD interaction (+ $p < 0.05$) two-way RM ANOVA; *, ** $p < 0.05, 0.01$ vs. naïve, # $p < 0.05$ vs. BL, Bonferroni posttest.

NMS increased susceptibility to TNBS

To determine whether NMS increased susceptibility to experimental colitis, two different concentrations of TNBS were applied intracolonically. Application of 2mg/mouse TNBS did not significantly alter body weight, survival, or colorectal sensitivity of naïve or NMS mice, compared to saline-treated counterparts, over the 4 days immediately following treatment (Figure 3.2). However, 5mg/mouse TNBS resulted in significant weight loss in NMS mice, compared to both 5mg TNBS-treated naïve mice and saline-treated NMS mice (Figure 3.2B) and also decreased survival rate in NMS mice, compared to 5mg TNBS-treated naïve mice (16.7% ± 15.2 vs. 100%; $p = 0.0006$, Log-rank Mantel-Cox test). Neutrophil activation was measured by MPO activity and NMS mice that were separated for only the first 14 days of life and treated with 5mg TNBS had significantly higher MPO at 4 d post-treatment compared to baseline NMS measurements (Table 3.2). NMS had a significant impact on colorectal sensitivity in both the saline-treated and 5mg TNBS-treated groups; however, TNBS treatment showed no significant impact on colorectal sensitivity in either NMS or naïve mice (Figure 3.2A).

NMS enhanced acute WAS-induced colorectal hypersensitivity

Colorectal sensitivity was measured prior to and 1 d after either a single exposure to 1 h of WAS (1d WAS) or 7 consecutive daily exposures to 1 h of WAS (7d WAS) to determine susceptibility to acute or chronic stress, respectively. The VMR during the entire CRD series was significantly higher in NMS mice exposed to 1d WAS compared to their baseline measurements (Figure 3.3A). In particular, the VMR at 60 and 75 mmHg was significantly higher in NMS mice after 1d WAS than both their baseline measurements and that of their naïve counterparts (Figure 3.3A). Exposure to 1d WAS did not significantly impact the VMR of naïve mice (Figure 3.3A). Exposure to 7d WAS did not significantly impact the VMR of either naïve or NMS mice (Figure 3.3B).

Figure 3.2 Colonic sensitivity and body weight after TNBS treatments

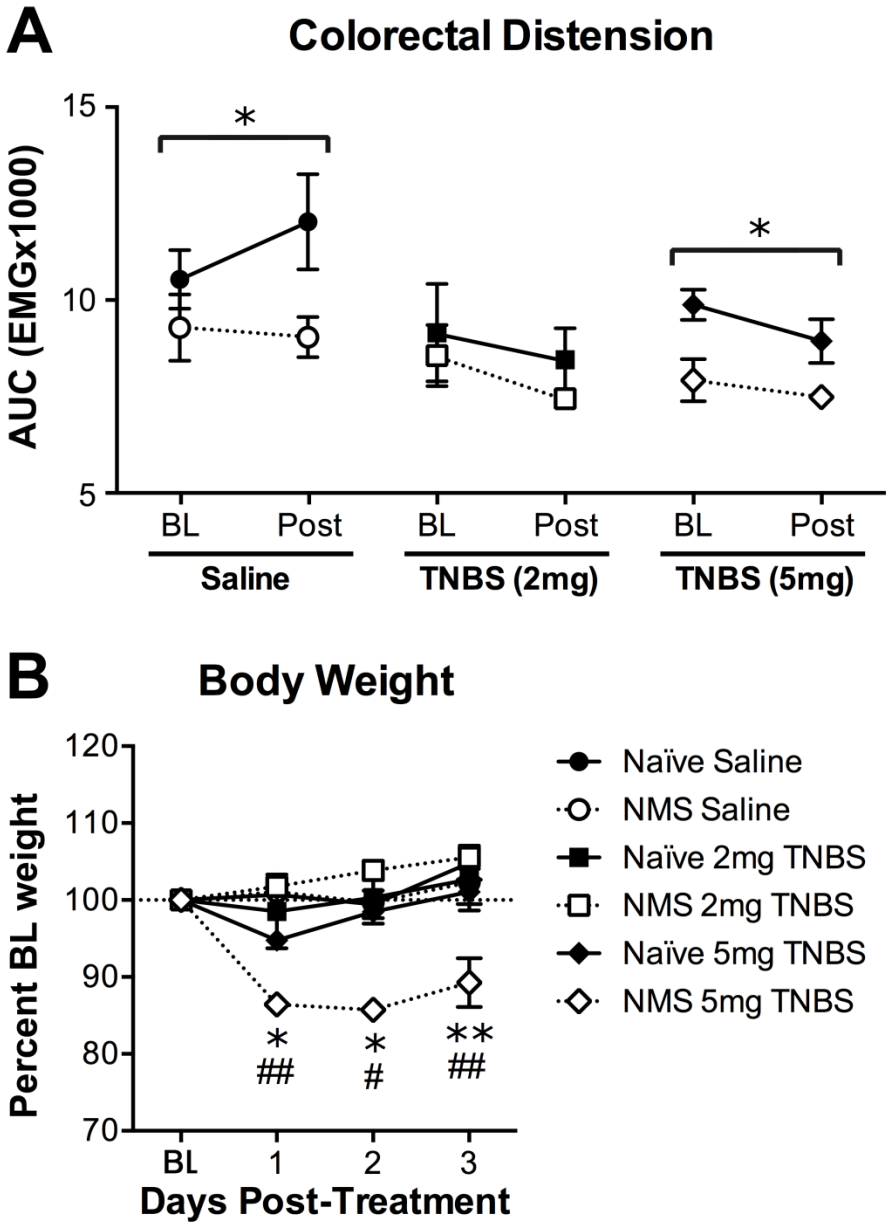

Figure 3.2 Colonic sensitivity and body weight after TNBS treatments. Mice that underwent neonatal maternal separation (NMS) were dose-dependently susceptible to intracolonic administration of trinitrobenzene sulfonic acid (TNBS). Mice were assessed for colorectal sensitivity and weighed to determine baseline (BL) measurements and then treated with saline, 2 mg TNBS, or 5 mg TNBS. Body weight was measured daily and colorectal distension (CRD) was performed on day 4 post-TNBS. **A)** The total electromyographic (EMG) activity is expressed as the area under the curve (AUC) for the entire CRD pressure series. No treatment effects were observed for saline, 2 mg TNBS, or 5 mg TNBS; however, NMS significantly impacted VMR in the saline-treated and 5 mg TNBS-treated groups. **B)** NMS mice given 5 mg TNBS lost significantly more body weight over three subsequent days than either naïve mice treated with 5 mg TNBS or saline-treated NMS mice. Brackets indicate a significant effect of NMS (*$p < 0.05$), two-way RM ANOVA; *, **$p < 0.05$, 0.01 vs. naïve, #, ##$p < 0.05$, 0.01 vs. saline, Bonferroni posttest.

Table 3.2 Myeloperoxidase activity following trinitrobenzene sulfonic acid treatment

	Naïve	NMS‡
Baseline (n=3)	0.852 ± 0.20	0.606 ± 0.25
1d post (n=4)	6.43 ± 1.64	8.06 ± 0.77
4d post (n=4)	4.75 ± 1.81	12.12 ± 3.81##

Myeloperoxidase (MPO) activity (expressed as U mg^{-1} tissue weight) was measured in distal colon from naïve and NMS mice prior to (baseline) and following intracolonic instillation of 5 mg trinitrobenzene sulfonic acid (TNBS). Treatment with TNBS significantly increased MPO levels in both groups (p=0.0071, two-way ANOVA). Naïve MPO levels peaked at 1d post-TNBS, while NMS MPO levels continued to increase at 4d post-TNBS. ##$p < 0.01$ vs. baseline, Bonferroni posttest. ‡ denotes NMS (neonatal maternal separation) mice that were separated from postnatal day 1-14.

Figure 3.3 Visceromotor response to colorectal distension after WAS

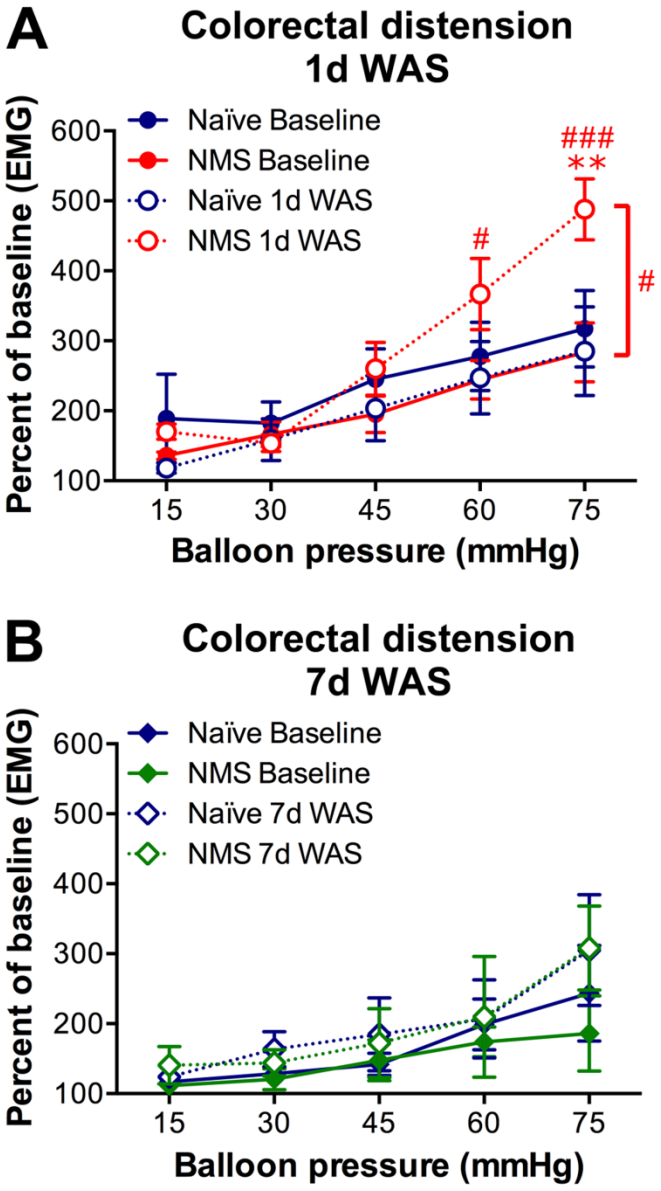

Figure 3.3 Visceromotor response to colorectal distension after WAS. Acute, but not chronic, exposure to water avoidance stress (WAS) selectively increased colorectal sensitivity in NMS mice. **A)** The electromyographic (EMG) activity of the abdominal musculature was measured to determine the visceromotor response (VMR) to colorectal distension (CRD) both prior to (baseline) and 1 day after a single exposure to WAS (1d WAS) in naïve and NMS mice. Exposure to 1d WAS significantly increased VMR of NMS mice compared to their baseline measurements. This was particularly evident at the highest balloon pressures applied, 60 and 75 mmHg. **B)** Chronic daily exposure to WAS (7d WAS) had no impact on VMR during CRD in either naïve or NMS mice. Brackets indicate a significant effect of WAS (#$p < 0.05$), two-way RM ANOVA; **$p < 0.01$ vs. naïve, #, ###$p < 0.05$, 0.001 vs. baseline, Bonferroni posttest.

NMS blunted TNBS and WAS-induced inflammatory gene expression and increased CRF receptor expression in the colon

The impact of NMS, TNBS, and WAS on pro-inflammatory gene expression in the distal colon was evaluated by RT-PCR. Treatment with 5mg TNBS induced a trend toward increased IL-6 and Artemin mRNA levels in the distal colon of naïve mice, which was not present in NMS mice (Figure 3.4A). Indeed, artemin mRNA levels were significantly lower in NMS colon compared to naïve, particularly following TNBS treatment (Figure 3.4A). Exposure to 1d WAS significantly increased IL-6 mRNA levels in naïve colon and the mRNA levels of IL-6 trended toward a significant increase in NMS mice, (p = 0.0525, two-way ANOVA; Figure 3.4B). Exposure to 1d WAS had no impact on artemin mRNA levels in the colon of either naïve or NMS mice (Figure 3.4B).

To determine the impact of 1d WAS on peripheral CRF receptor levels, Western blot was performed to measure CRF_1 and CRF_2 protein levels in the distal colon. Protein levels of CRF_1 in the distal colon were not affected by NMS or 1d WAS (Figure 3.4C). In contrast, 1d WAS exposure significantly increased CRF_2 protein levels overall, specifically in naïve colon post-1d WAS compared to baseline levels (Figure 3.4C). CRF_2 protein levels were increased, though not significantly, in NMS colon both at baseline and post-1d WAS compared to baseline naïve levels (Figure 3.4C).

Figure 3.4 Colonic molecular changes after TNBS or WAS treatments

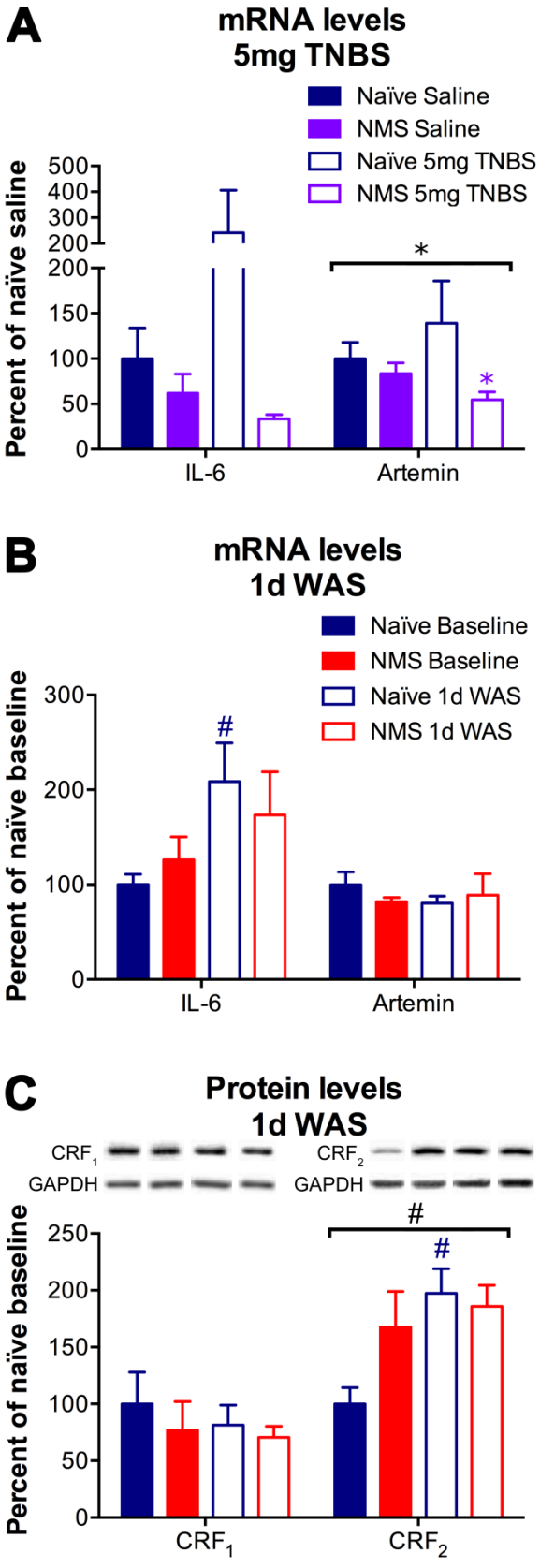

Figure 3.4 Colonic molecular changes after TNBS or WAS treatments. Neonatal maternal separation (NMS) alters localized gene and protein expression changes in response to inflammation and acute stress. **A)** Real-time PCR was performed on mRNA from naïve and NMS distal colon following saline or 5 mg trinitrobenzene sulfonic acid (TNBS) treatment. A non-significant increase in interleukin (IL)-6 mRNA levels was observed in naïve colon following TNBS treatment, with a corresponding decrease in both IL-6 and artemin mRNA levels in NMS colon. **B)** Exposure to 1d WAS significantly increased IL-6 mRNA levels in naïve colon and NMS effected a trend toward increased IL-6 mRNA levels ($p = 0.0525$, two-way ANOVA). Artemin mRNA levels were unaffected by either NMS or WAS. **C)** Representative Western blots are shown for CRF_1, CRF_2, and corresponding GAPDH protein with bands at 55, 49, and 35 kD, respectively. Exposure to 1d WAS had no effect on CRF_1 protein levels in either naïve or NMS colon. At baseline, CRF_2 protein levels were increased in NMS colon and exposure to 1d WAS significantly increased CRF_2 protein levels, particularly in naïve colon. Brackets indicate a significant effect of NMS (*$p < 0.05$) or WAS (#$p < 0.05$), two-way RM ANOVA; *$p < 0.05$ vs. naïve, #$p < 0.05$ vs. baseline, Bonferroni posttest.

Chapter IV: Results: NMS-induced perigenital hypersensitivity and function in response to acute adult stress

Experimental design

All experimental naïve and NMS males were subjected to acute water avoidance stress (WAS) as adults (20 weeks of age) and assessed for perigenital sensitivity and micturition activity 1-day pre-, 1-day post-, and 8-days post-WAS. *In vivo* experiments were conducted within the first six hours of the light cycle. Tissues were collected for *in vitro* analysis immediately following 8d post-WAS reassessment. Control naïve and NMS males were undisturbed in their home cages aside from normal husbandry procedures and tissues were collected between 10 and 12 weeks of age.

NMS, but not WAS, induced perigenital hypersensitivity and increased micturition output and frequency

Male mice that did (NMS) or did not (naïve) undergo neonatal maternal separation were assessed as adults for perigenital sensitivity or micturition patterning before and following WAS exposure. At baseline, NMS mice exhibited a significantly lower perigenital mechanical withdrawal threshold than naïve mice (Figure 4.1A). At 1d and 8d post-WAS, NMS males continued to exhibit an average perigenital mechanical withdraw threshold that was significantly lower than naïve counterparts, although WAS had no significant impact on perigenital sensitivity in either group (Figure 4.1A). At baseline, NMS mice had a significantly higher total voided volume compared to naïve mice, as well as at 1d post-WAS (Figure 4.1B). No difference in total voided volume was observed between naïve and NMS mice at 8d post-WAS; however, NMS output was significantly lower than at baseline (Figure 4.1B). NMS mice also had a significantly higher number of voids at baseline than naïve mice (Figure 4.1C) and no differences were observed in number of voids between naïve and NMS mice at 1d or 8d post-WAS (Figure 4.1C). NMS mice had significantly increased fecal output at baseline compared to naïve (Table 4.1).

Figure 4.1 Perigenital mechanical withdrawal thresholds and micturition patterns after WAS

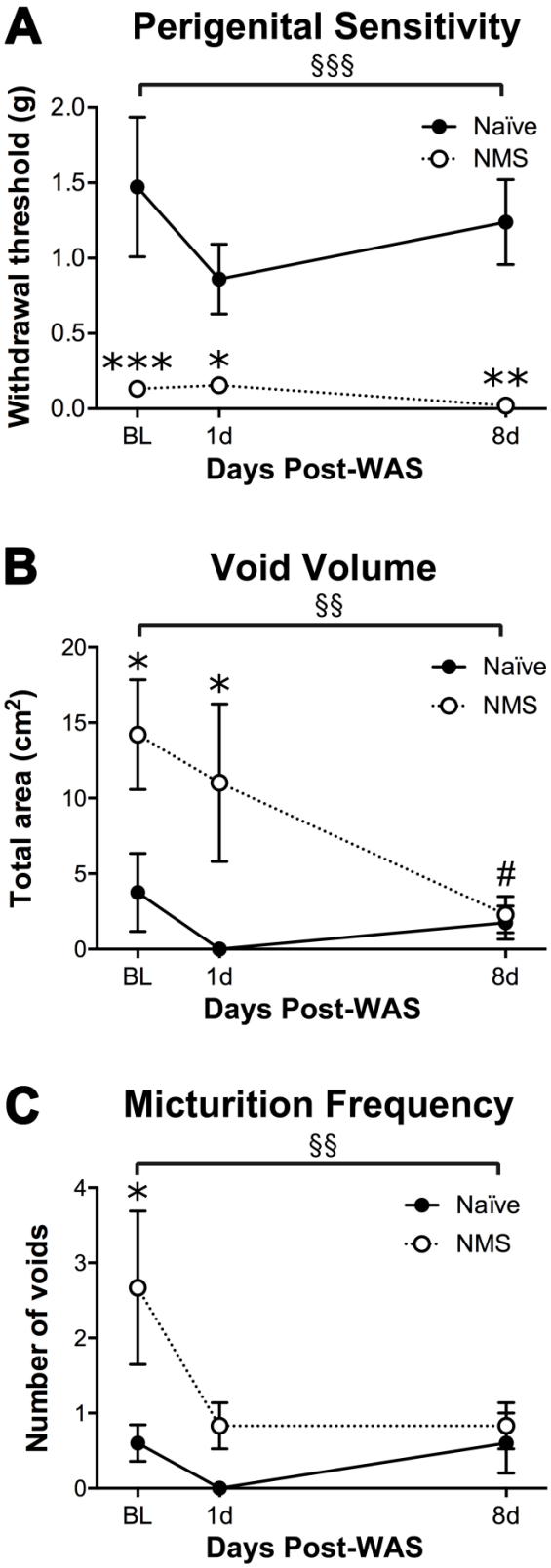

Figure 4.1 Perigenital mechanical withdrawal thresholds and micturition patterns after WAS**.** NMS exposure had a more significant impact on perigenital mechanical sensitivity and micturition function than WAS exposure. **A)** Perigenital mechanical withdraw thresholds were significantly lower for male mice exposed to NMS across all time points. **B)** NMS males displayed a significantly higher average for void volume than naïve males at baseline and 1d post-WAS. At 8d post-WAS, NMS males averaged smaller void areas compared to their respective baseline. **C)** Significant differences in micturition frequency were only observed at baseline, when NMS males averaged more void events compared to naïve males. Brackets indicate significant effect of NMS (§§, §§§$p < 0.01, 0.001$), two-way RM ANOVA; *, **, ***$p < 0.05, 0.01, 0.001$ vs. naïve, #$p < 0.05$ vs. NMS-baseline, Bonferroni posttest (n = 6).

Table 4.1 Correlation between urinary and gastrointestinal outputs following WAS

Treatment group	Number of pellets	Correlation with total urinary output
Naïve baseline	1.00 ± 0.45	r = 0.9393, *p* = 0.0178
Naïve 1d post-WAS	1.67 ± 0.42	N.D.
Naïve 8d post-WAS	1.17 ± 0.60	r = 0.9457, *p* = 0.015
NMS baseline	3.83 ± 0.70*	r = 0.7545, *p* = 0.0830
NMS 1d post-WAS	2.00 ± 0.93	r = 0.1887, *p* = 0.7203
NMS 8d post-WAS	2.17 ± 0.48	r = -0.02184, *p* = 0.9672

The number of pellets were counted following the one-hour micturition analysis test and averaged within groups and correlated with total urinary output within mice. NMS significantly increased gastrointestinal output compared to naïve mice both overall (*p* = 0.0164, two-way RM ANOVA) and at baseline (* *p* = 0.003, Fisher's LSD). Only naïve mice at baseline and 8d post-WAS displayed a significant correlation between gastrointestinal and urinary output (Pearson's correlation). N.D. = not detected.

Interestingly, the total urine output significantly correlated with fecal output at baseline and 8d post-WAS only in naïve mice.

WAS exposure increased sucrose preference for both naïve and NMS males

Mice were acclimated to two liquid-choice cages for 48 hours, both bottles filled with standard drinking water, before one bottle was replaced with 1% sucrose solution and mice were assessed for baseline sucrose preference over 4 days prior to WAS exposure. After 1 hour WAS exposure, mice were returned to two-choice liquid preference cages and liquid consumption was recorded every 24 hours until 8d post-WAS, but reported in Figure 4.2 every 48 hours. Naïve and NMS males exhibited similar baseline sucrose preference, and did not significantly differ from one another throughout the duration of the study; however, time had a significant impact on percent sucrose intake after WAS exposure.

NMS and WAS had tissue-specific effects on mast cell degranulation inflammatory gene expression in urogenital tissues

Cryosections of bladder and prostate tissues were stained using toluidine blue-O to stain mast cells. Figure 4.3 A-D are representative images of stained prostate tissue. Intact and degranulated mast cells were quantified and the degranulated percentage was calculated. Consistent with our previous results [137], control NMS, as well as WAS-exposed, males displayed significantly higher percentages of degranulated mast cells in both bladder and prostate tissues compared to naïve counterparts. Interestingly, WAS exposure did not alter mast cell degranulation in bladder tissue, but did significantly increase mast cell degranulation in prostate tissue (Figure 4.3E).

Figure 4.2 WAS exposure increased sucrose preference for both naïve and NMS males

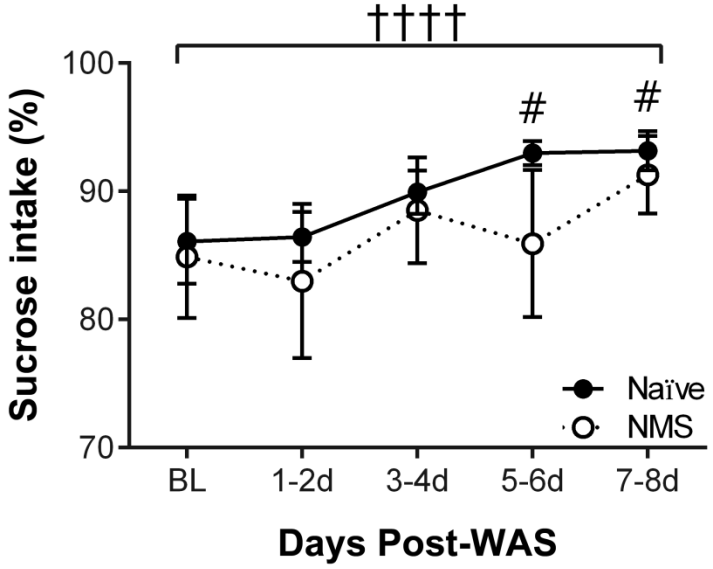

Figure 4.2 WAS exposure increased sucrose preference for both naïve and NMS males. Reported baseline sucrose preference was averaged over the 4-day baseline period. Data was reported as a percentage of sucrose consumed over a 2-day period to control for a possible side preference, as bottles were switched every 24 hours. Sucrose preference for NMS males did not differ from naïve controls. Naïve sucrose consumption was significantly increased from baseline during the last two 48-hour periods. Bracket indicates significant effect of time (††††$p < 0.0001$); two-way RM ANOVA, #$p < 0.05$ vs. naïve-baseline, Bonferroni posttest (n = 5-6).

Figure 4.3 Urogenital mast cell degranulation after WAS

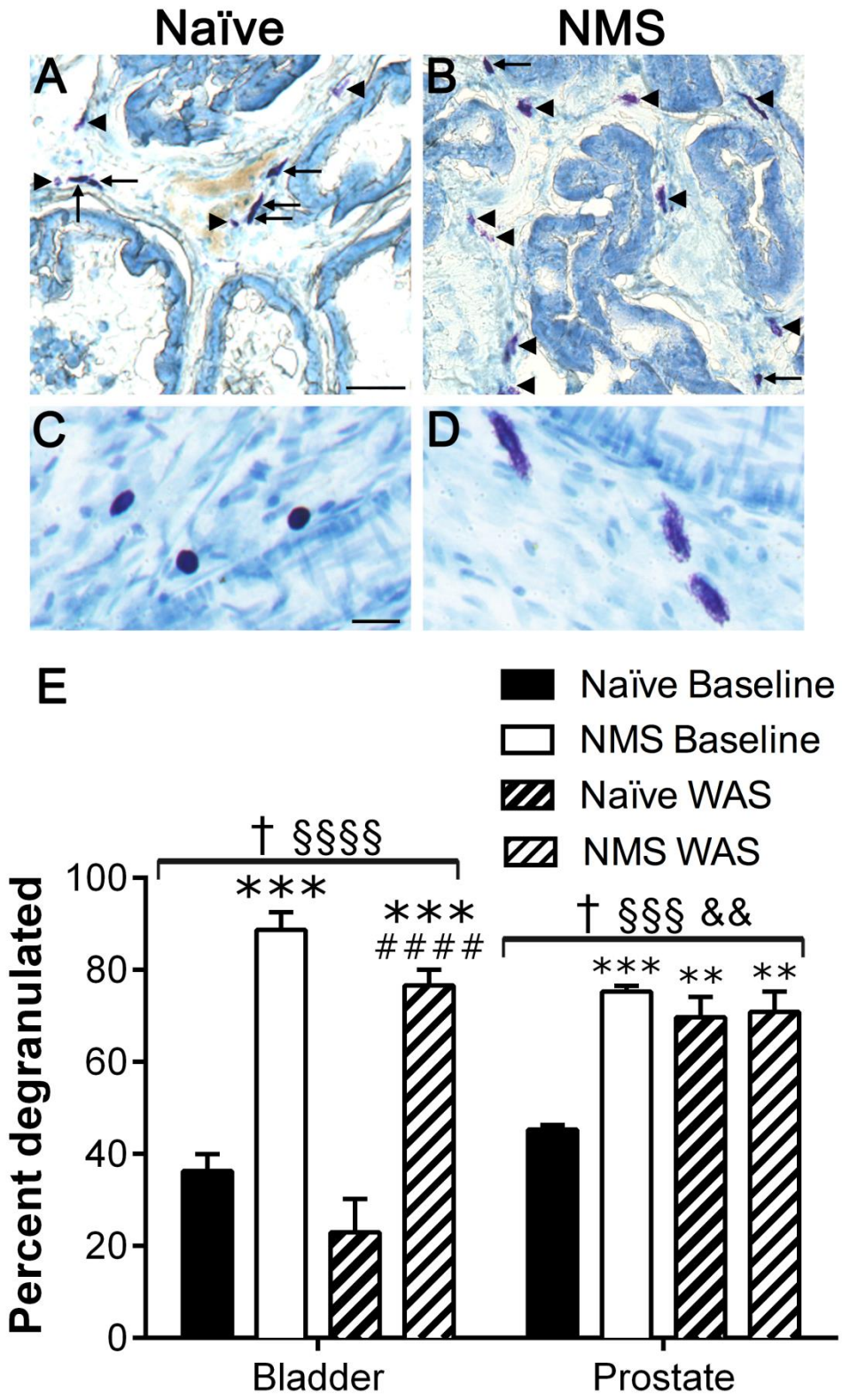

Figure 4.3 Urogenital mast cell degranulation after WAS. Acidified toluidine blue was used to visualize tryptase granules and calculate the percentage of activated/degranulated mast cells in cryostat sections of bladder and prostate tissues. Representative photomicrographs are shown of toluidine blue-stained sections from naïve **(A)** and NMS **(B)** prostate with arrows indicating intact (non-degranulated) and arrowheads indicating activated (degranulated) mast cells. Higher magnification images from naïve **(C)** and NMS **(D)** bladder are shown to illustrate histological differences from non-degranulated **(C)** and degranulated **(D)** mast cells. **E)** Bladders from mice subjected to NMS with or without WAS exposure exhibited significantly higher percentages of degranulated mast cells compared to baseline naïve males; though, WAS exposure did not affect bladder mast cell degranulation of naïve mice. Mast cell degranulation percentages were found to be increased in prostate tissues of NMS males with or without exposure to WAS, as well as naïve males exposed to WAS. Scale bars equal 100µm **(A-B)** and 40µm **(C-D)**. **E:** Brackets indicate significant effect of NMS (§§§, §§§§$p < 0.001, 0.0001$), WAS (†$p < 0.05$), and/or NMS-WAS interaction (&&$p < 0.01$), two-way ANOVA; **, ***$p < 0.01$, 0.001 vs. naïve-baseline, ####$p < 0.0001$ vs. naïve-WAS, Bonferroni posttest (n = 3-4).

Tissue-specific differences were also observed for inflammatory gene expression changes in response to NMS and/or WAS exposure. RT-PCR was used to measure interleukin-6 (IL-6), interleukin-10 (IL-10), stem cell factor (SCF), artemin, nerve growth factor (NGF), and monocyte chemoattractant protein-1 (MCP-1) mRNA levels in the bladders and prostates of naïve and NMS male mice with or without exposure to WAS. In bladder, WAS had a significant effect on increased artemin mRNA levels. NGF expression was significantly affected by NMS, as well as an NMS/WAS interaction effect. NMS-WAS males displayed the greatest increase of NGF mRNA levels; significantly greater than both naïve and NMS controls. An NMS/WAS interaction effect also had a significant impact on increased MCP-1 mRNA levels (Figure 4.4A). Similar to bladder values, prostatic IL-6 and IL-10 were not altered by NMS or WAS exposure. Expression of SCF was not altered in bladders across all four groups; however, in the prostates, decreases in SCF expression were attributed to NMS and NMS/WAS interaction effects. Only NMS controls expressed significantly less SCF mRNA than naïve controls. Both NMS control and WAS groups displayed significantly lower artemin mRNA levels. For all four groups, NMS had a significant impact on decreased prostatic artemin mRNA expression levels, whereas WAS had a significant impact on increases in MCP-1 (Figure 4.4B).

WAS disrupted regulatory gene expression in central structures of the HPA axis

To determine the impact of NMS and WAS on gene expression within central structures involved in the regulation and output of the HPA axis, mRNA levels of corticotropin releasing factor (CRF), urocortin 2 (Ucn2), CRF receptor 1 (CRF_1), CRF_2, glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) in hypothalamus, amygdala, and hippocampus of mice were determined by RT-PCR. WAS exposure had a significant impact on decreases in CRF, Ucn2, GR, and MR mRNA expression in hippocampi. CRF and MR were particularly low for both naïve and NMS WAS-exposed groups, whereas only naïve-WAS males displayed a statistically lower level of Ucn2 mRNA than controls (Figure 4.5A). In contrast, WAS had a

Figure 4.4 Neuroimmune profiles of urogenital tissues after WAS

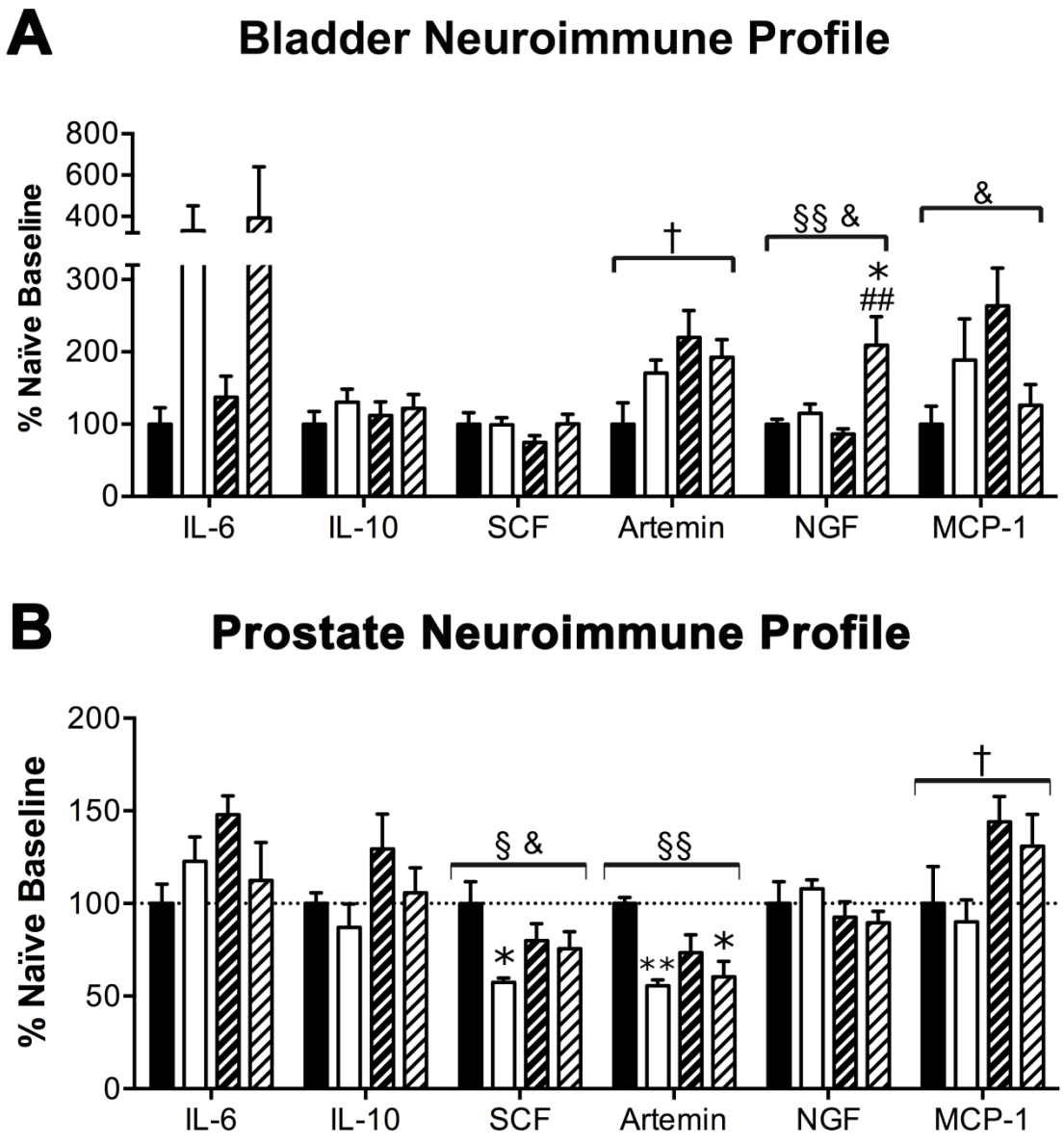

Figure 4.4 Neuroimmune profiles of urogenital tissues after WAS**.** **A)** Expression levels of nerve growth factor (NGF) in the bladders of NMS-WAS males were elevated compared to both naïve-baseline and naïve-WAS males. **B)** Baseline NMS stem cell factor (SCF) and both baseline and WAS NMS artemin prostatic gene expression levels were significantly lower than respective naïve baselines. Brackets indicate significant effect of NMS (§, §§$p < 0.05, 0.01$), WAS (†$p < 0.05$), and/or NMS-WAS interaction (&$p < 0.05$), two-way ANOVA; *, **$p < 0.05, 0.01$ vs. naïve-baseline, ##$p < 0.01$ vs. naïve-WAS, Bonferroni posttest (n = 5).

Figure 4.5 Gene expression changes within limbic structures after WAS

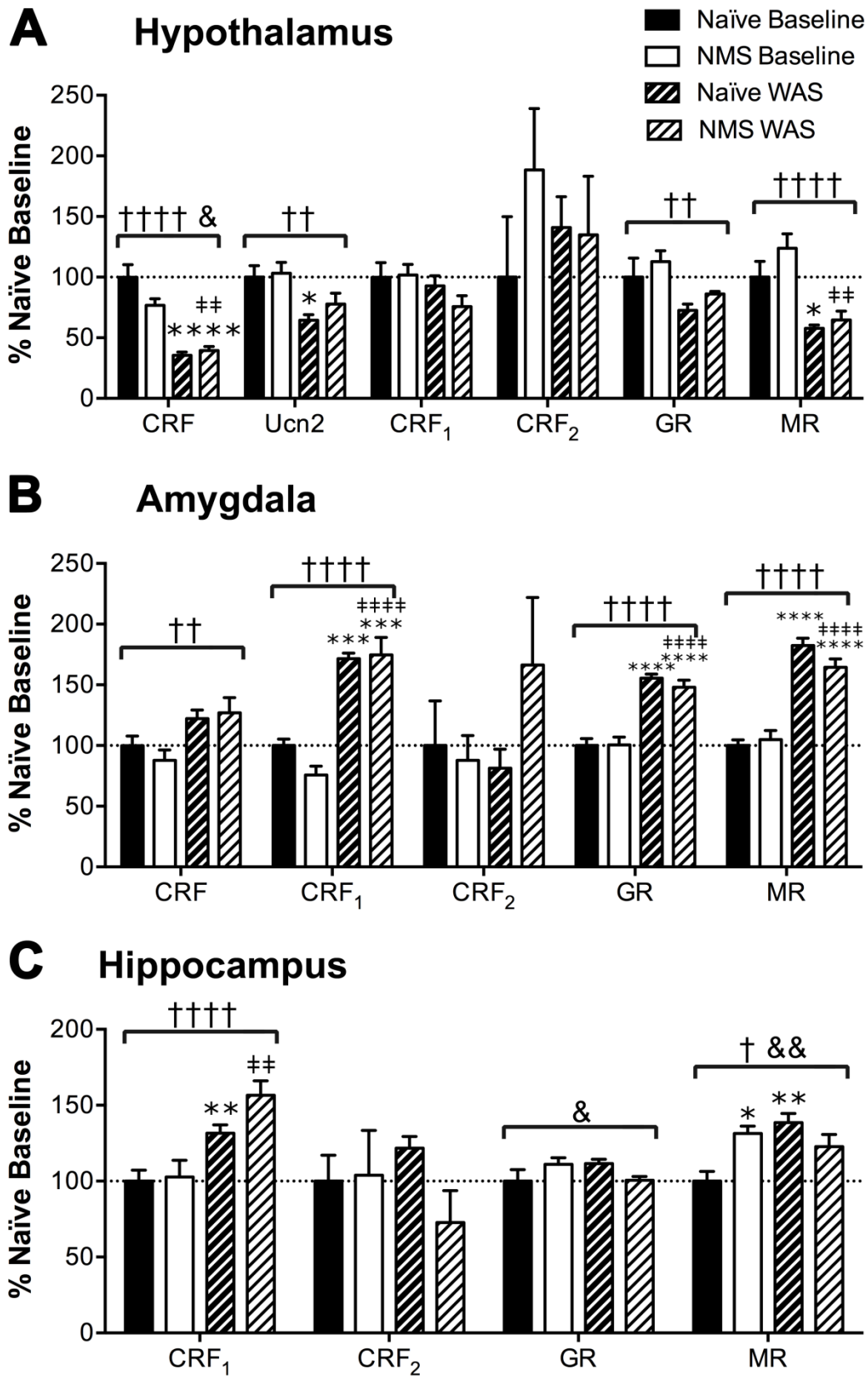

Figure 4.5 Gene expression changes within limbic structures after WAS. Hypothalamic expression of genes associated with the HPA axis were generally downregulated after WAS exposure for both naïve and NMS male mice **(A)**. Gene expression in the amygdala, on the other hand, was largely upregulated following WAS **(B)**. Gene expression in the hippocampus was variably altered; CRF_1 was upregulated for both NMS and naïve males after WAS, whereas MR expression was increased for baseline NMS and WAS naïve males **(C)**. Bracket indicates significant effect WAS (†, ††, ††††p < 0.05, 0.01, 0.0001) and/or NMS-WAS interaction (&, &&p < 0.05, 0.01), two-way ANOVA; *, **, ***, ****p < 0.05, 0.01, 0.001, 0.0001 vs. naïve-baseline, ǂǂ, ǂǂǂǂp < 0.01, 0.0001 vs. NMS-baseline, Bonferroni posttest (n = 5-6).

significant impact on increases in CRF, CRF_1, GR, and MR mRNA levels in amygdalae. WAS-exposed naïve and NMS groups showed significantly higher levels of CRF_1, GR, and MR mRNA compared to their baseline counterparts (Figure 4.5B). A similar trend was observed for CRF_1 mRNA levels in hippocampi. Increases in GR were statistically attributed to an NMS/WAS interaction effect, and MR to both WAS and an interaction effect. Additionally, NMS controls and naïve WAS-exposed males exhibited similar increases in MR mRNA expression (Figure 4.5C).

Stress decreased downstream HPA axis output

To complement our central HPA axis-associated mRNA level investigation, we used Western blot assay to measure peripheral CRF_1 and CRF_2 protein expression, as well as ELISA analysis to measure serum corticosterone (CORT) concentrations following NMS and/or WAS. Bladder CRF_1 protein expression was significantly influenced by an NMS/WAS interaction (Figure 4.6A). CRF_2 protein levels, on the other hand, were not significantly altered in either the bladder or the prostate across all four groups (Figure 4.6A and B). Prostates of NMS-WAS mice exhibited significantly lower CRF_1 concentrations compared to NMS-baseline concentrations, but did not significantly differ from either naïve group. The effect of WAS and an NMS-WAS interaction effect trended toward significance ($p = 0.0616$ and $p = 0.0846$, respectively). When analyzed by student's t-test, NMS-baseline CRF_1 concentrations trended toward a significant increase compared to naïve-baseline concentrations ($p = 0.0769$) (Figure 4.6B). Baseline NMS males displayed significantly lower concentrations of serum CORT compared to naïve counterparts. WAS-exposed groups displayed similar concentrations, but were not significantly different from either control group (Figure 4.6C).

Figure 4.6 Western blot and serum CORT ELISA analysis after WAS

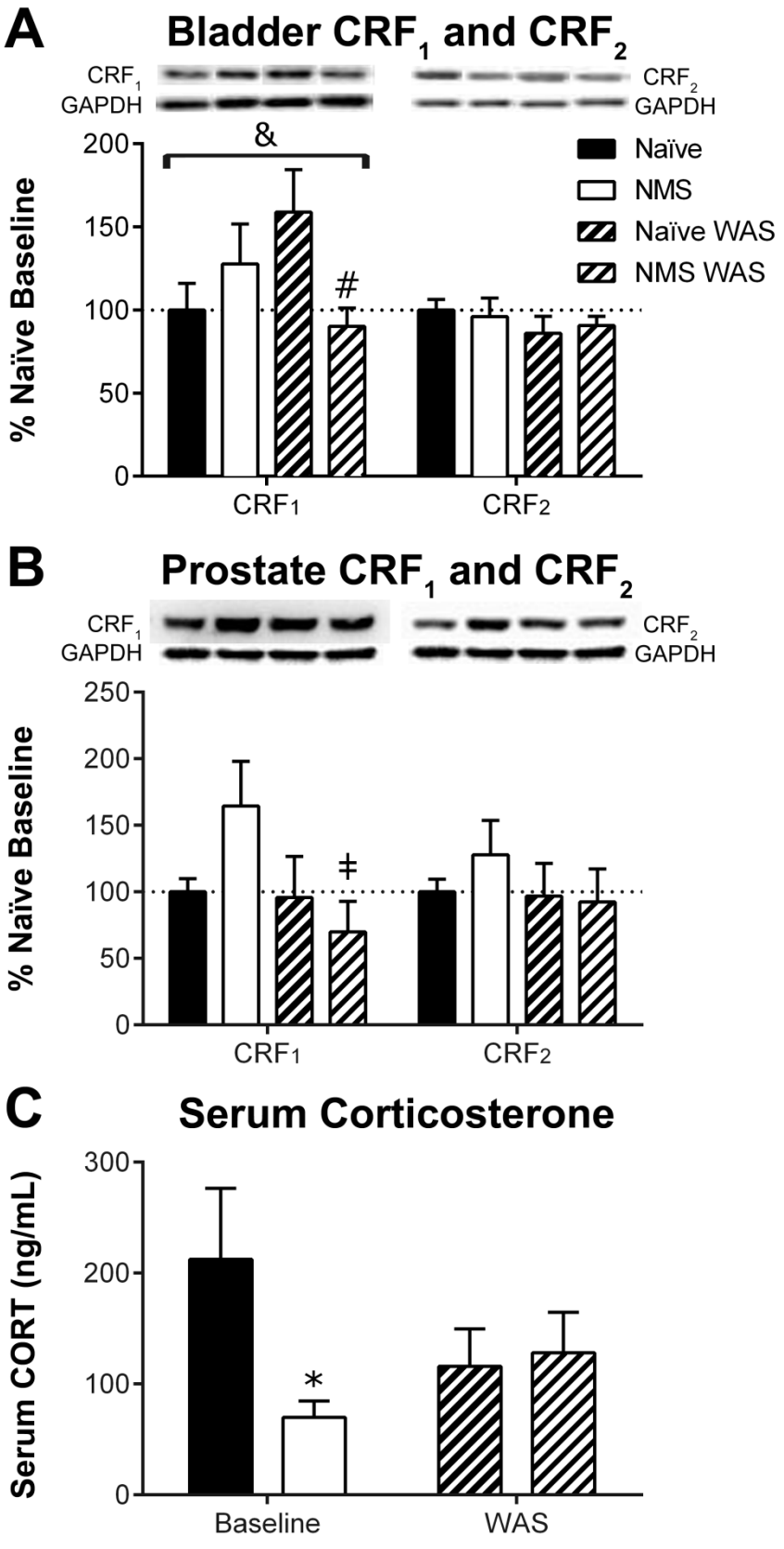

Figure 4.6 Western blot and serum CORT ELISA analysis after WAS. Representative Western blots are shown for CRF_1, CRF_2, and corresponding GAPDH protein with bands at 55, 49, and 35kD, respectively. **A)** Bladders of NMS males exposed to WAS displayed significantly lower CRF_1 protein levels than those of naïve-WAS males. Bladder CRF_2 levels were not observed to be altered as a result of NMS nor WAS exposure. **B)** NMS-WAS prostates also exhibited significantly lower CRF_1 concentrations compared to NMS-baseline concentrations. The effect of WAS and an NMS-WAS interaction effect trended toward significance ($p = 0.0616$ and $p = 0.0846$, respectively). When analyzed by student's t-test, NMS-baseline CRF_1 concentrations trended toward a significant increase compared to naïve-baseline concentrations ($p = 0.0769$). For CRF_2 concentrations, on the other hand, did not significantly differ across all four groups. **C)** Males subjected to NMS exhibited lower concentrations of serum corticosterone (CORT) compared to baseline naïve males. Exposure to WAS did not significantly influence serum CORT concentrations. Brackets indicate significant effect of NMS/WAS interaction (&$p < 0.05$), two-way ANOVA, *p <0.05 vs. naïve-baseline, #p <0.05 vs. naïve-WAS, ǂ$p < 0.05$ vs. NMS-baseline; Bonferroni posttest (n = 5-6).

Chapter V: Results: Exercise interventions

Experimental design

We investigated the therapeutic potential of two voluntary wheel running intervention paradigms to attenuate neonatal maternal separation (NMS)-induced behavior and corresponding molecular changes: 1) early exercise beginning at 4 weeks of age (-Eex) and 2) late exercise beginning at 8 weeks of age (-Lex). Early and late sedentary counterparts are identified by –Esed and –Lsed, respectively. Early-exercised mice were pair housed with free access to a running wheel, whereas late-exercised mice were singly housed with a running wheel. Behavior assessments for early-sedentary and -exercised males were performed once at 8 weeks of age following introduction to cages equipped with running wheels or sedentary living conditions. Late-sedentary and -exercised males were assessed for baseline behavioral measurements at 8 weeks of age and reassessed at 12 weeks and/or 16 weeks of age. Experimental timelines can be found in Figure 5.1.

Early exercise prevented NMS-induced perigenital hypersensitivity

Consistent with our previous results [137], NMS induced perigenital hypersensitivity. For both NMS-Esed and baseline NMS-Lex measurements, mice exhibited significantly lower withdrawal thresholds in response to mechanical, monofilament stimulation to the scrotum compared to their naïve counterparts (Naïve-Esed and Naïve-Lex baseline, respectively) (Figure 5.1). Adolescent access to running wheels prevented this observed NMS-induced allodynia: Naïve-Eex and NMS-Eex males showed similar withdrawal thresholds that did not significantly differ from Naïve-Esed males. Additionally, early exercise and an NMS/exercise effect had a significant impact on perigenital mechanical sensitivity (Figure 5.1A). An NMS/exercise effect also significantly influenced withdrawal thresholds for late exercised males. After 8 weeks of running wheel exposure, Naïve-Lex and NMS-Lex withdrawal thresholds did not significantly differ; however, it is difficult to conclude late exercise reversed perigenital

Figure 5.1 Exercise paradigms and perigenital mechanical withdrawal thresholds

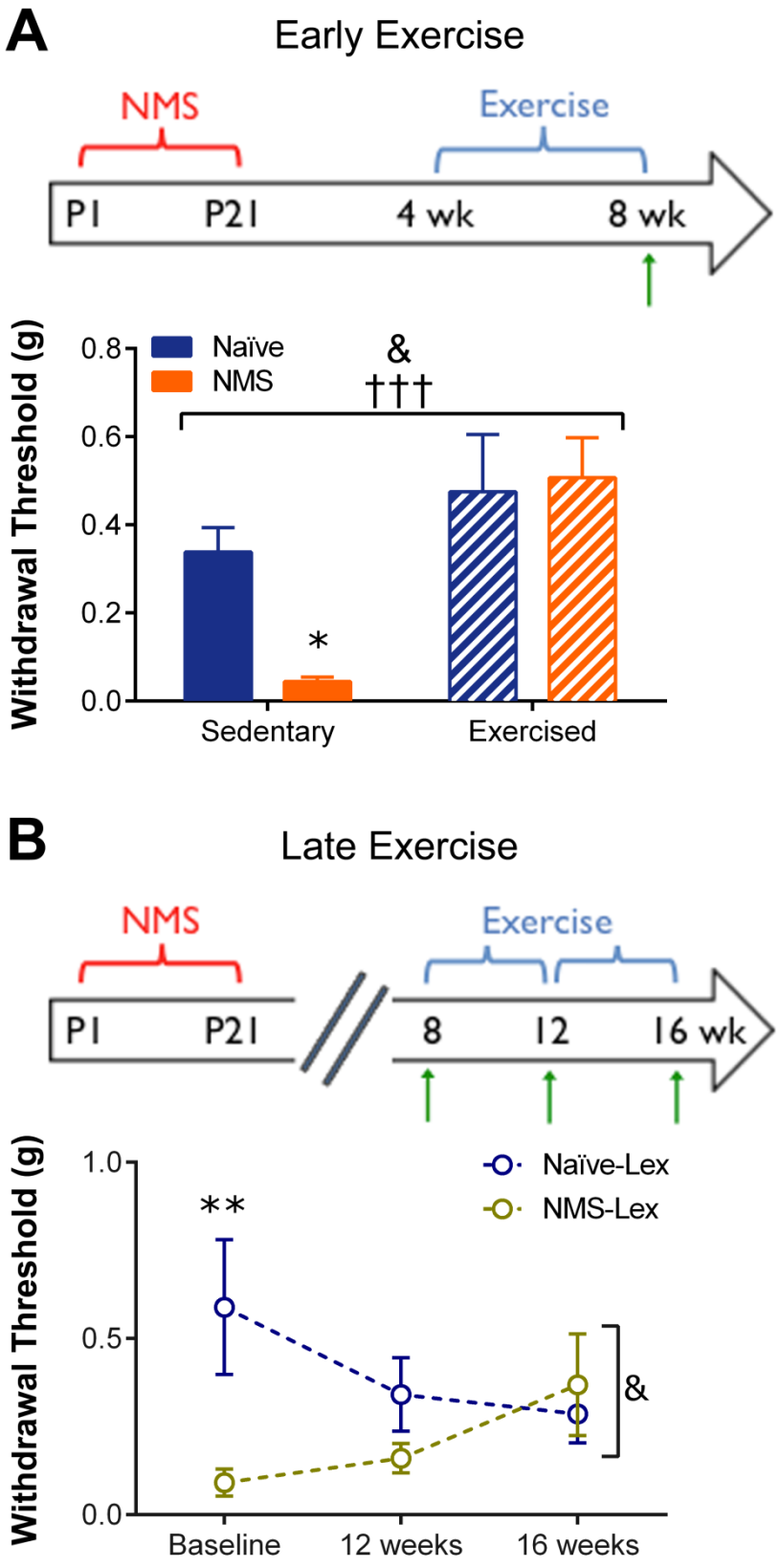

Figure 5.1 Exercise paradigms and perigenital mechanical withdrawal thresholds. NMS-induced perigenital mechanical hypersensitivity was prevented and reversed by voluntary wheel running. **A)** Sedentary NMS males displayed significantly lower perigenital withdraw thresholds to mechanical stimulation; however, this lowering of threshold was prevented by early voluntary wheel running. Pictured above is a schematic representation of the early exercise intervention timeline. **B)** Like sedentary NMS males of the early exercise study, NMS males before late exercise intervention exhibited significantly lower perigenital withdrawal thresholds. At the completion of 8 weeks of running wheel exposure, NMS thresholds increased to those similar to naïve. Experimental timeline can be found above line graph. **A:** Bracket indicates significant effect of exercise (†††$p < 0.001$) and interaction effect between NMS and early exercise exposure (&$p < 0.05$), two-way ANOVA, *$p < 0.05$ vs. sedentary naïve, Bonferroni posttest (n = 9-14). **B:** Bracket indicates significant NMS-late exercise interaction effect (&$p < 0.05$), two-way RM ANOVA, **$p < 0.01$ vs. baseline naïve, Bonferroni posttest (n = 7-8).

allodynia from these data. Where NMS-Lex thresholds increased over time, Naïve-Lex thresholds decreased, but not so much as to be significantly different from their baseline (Figure 5.1B).

Voluntary exercise normalized micturition patterns but increased bladder activity and output of NMS male mice

To measure micturition activity, mice were confined to a sheet of filter paper for one hour and the number and area of urine spots were recorded. Because chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) and interstitial cystitis (IC) patients experience up to 60 void events in a day in severe cases [60], we hypothesized NMS males to exhibit higher micturition frequencies compared to naïve males. Early-sedentary NMS males produced greater volumes of urine and void events than sedentary naïve males. We also predicted voluntary wheel running to attenuate any variations of micturition function. Following early exercise, NMS and naïve mice did not differ in void volume, void frequency, nor from sedentary controls (Figure 5.2A and C). In contrast, NMS males did not differ from naïve males in micturition output nor frequency at baseline, but NMS-Lex males did display significant decreases in both measures at 12 weeks of age following 4 weeks of running wheel access (Figure 5.2B and D).

The filter paper method described above, however, is a crude way to assess bladder function. For this reason, we also performed awake filling cystometry in sedentary and early-exercised naïve and NMS males. At a minimum of seven days after bladder catheter implantation surgery, animals were assessed for bladder function for 30 minutes during an infusion of saline at a rate of 20 μL/min. There were very few differences among the groups for the ten reported parameters. NMS-Eex males displayed significantly lower micturition frequencies (Figure 5.3B), but increased void masses (Figure 5.3G) and volumes of infused saline per micturition cycle (Figure 5.3H) compared to naïve-Esed males. NMS exposure and early exercise experience both had significant impacts on micturition frequency: NMS males

Figure 5.2 Micturition patterns following early and late exercise interventions

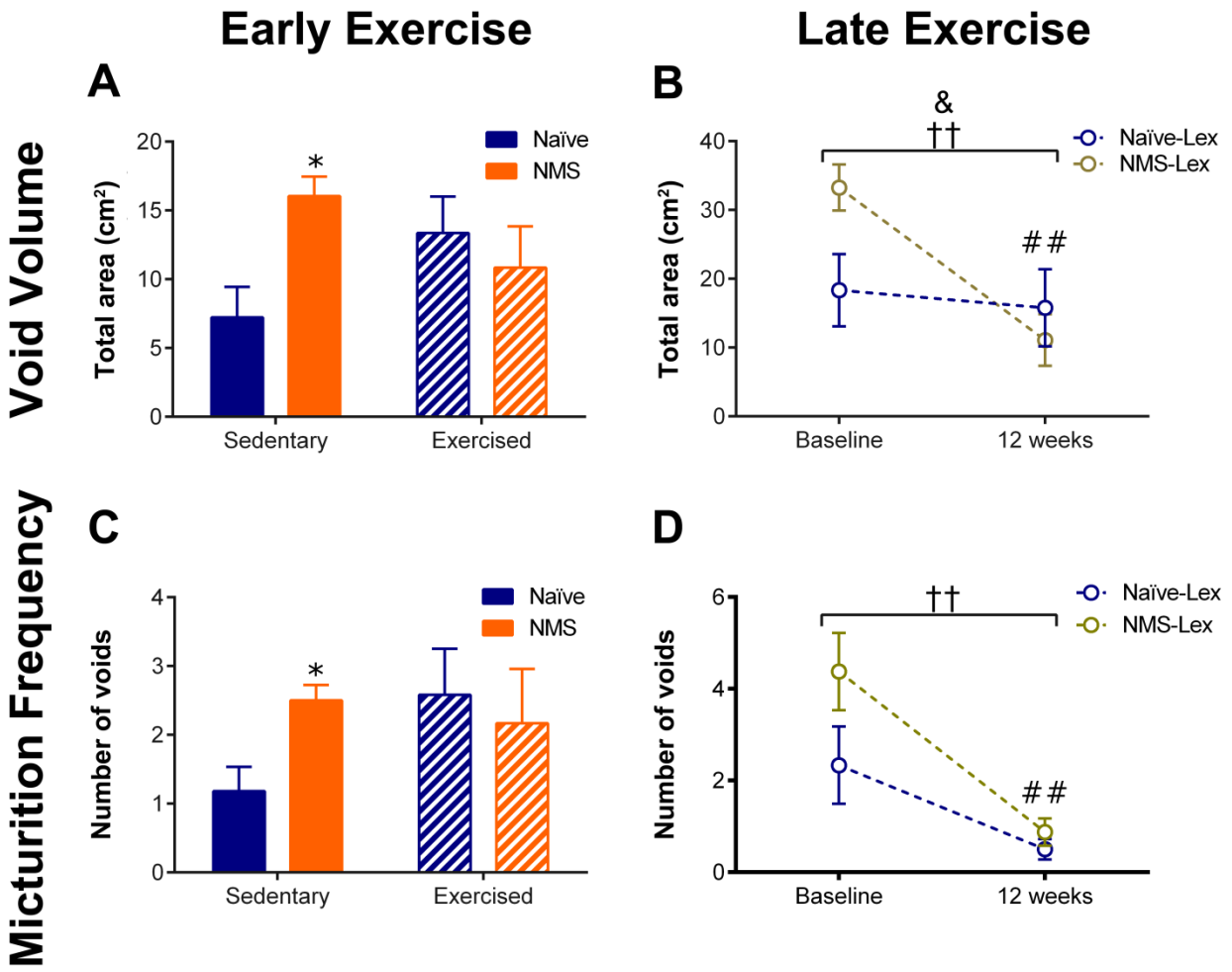

Figure 5.2 Micturition patterns following early and late exercise interventions. Sedentary NMS males exhibited greater void volumes **(A)** and higher number of void events **(C)** than sedentary naïve counterparts, but NMS and naïve mice did not display significantly different micturition patters following early exercise (**A** and **C**). At baseline, NMS-Lex males tended to produce greater void volumes **(B)** and higher void frequency **(D)**, but were not statistically different from naïve controls at baseline. After 4 weeks of voluntary wheel running, NMS and naïve males exhibited similar micturition patterns, and NMS-Lex void output and frequency significantly declined compared to their baseline measurements (**B** and **D**). **A and C:** unpaired, two-tailed student's t-test, **p* < 0.05 vs. naïve-Esed (n = 6-12). **B and D:** Brackets indicate significant effect of exercise (††p < 0.01) and NMS/exercise interaction (&*p* < 0.05); two-way RM ANOVA, ##*p* < 0.01 vs. NMS-Lex baseline, Bonferroni posttest (n = 8).

Figure 5.3 Effect of NMS and/or early exercise on urinary bladder cystometric assessment

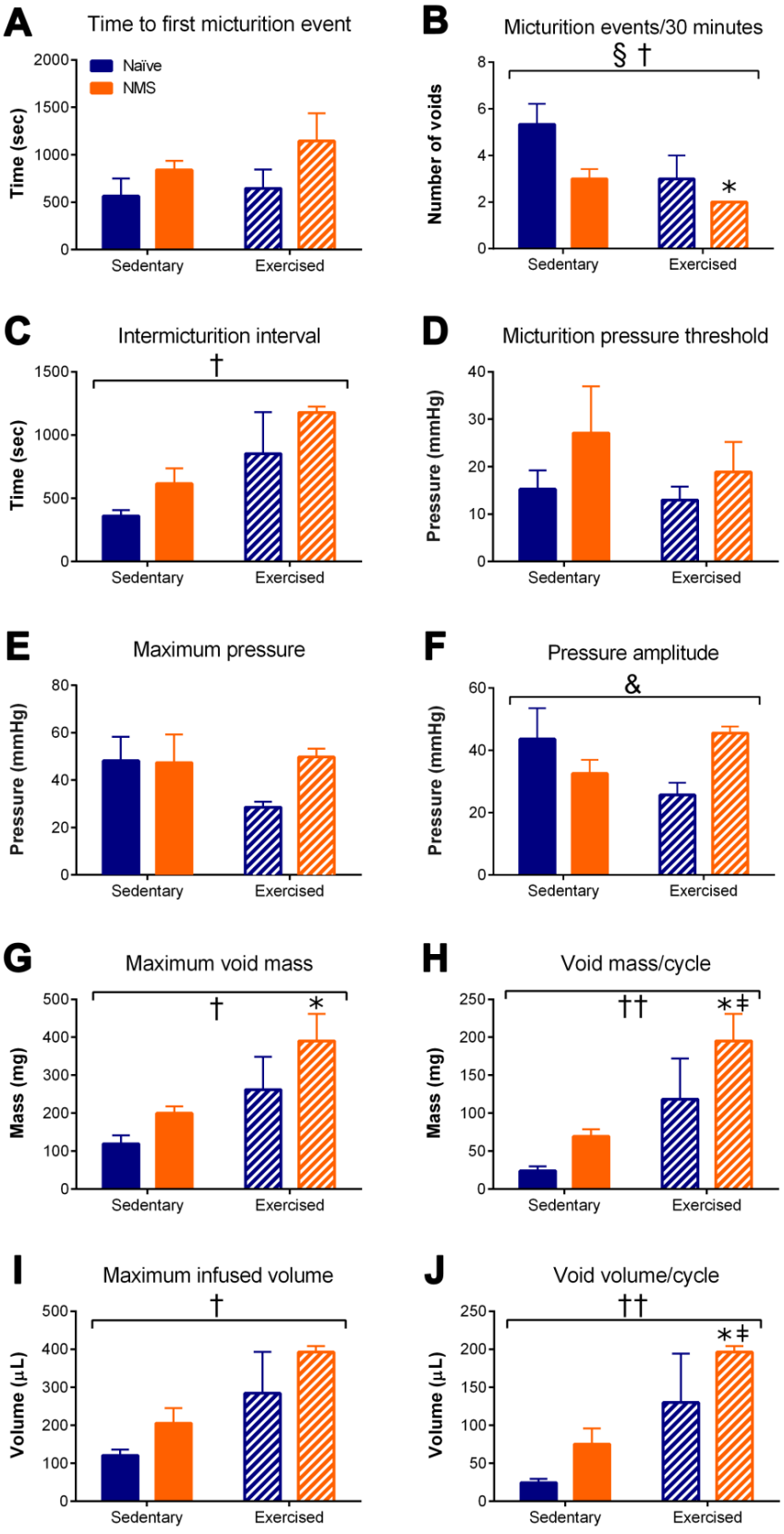

Figure 5.3 Effect of NMS and/or early exercise on urinary bladder cystometric assessment. **A)** Sedentary and exercised groups did not differ in the time to the first micturition event. **B)** Micturition frequency for sedentary NMS and exercised naïve males did not significantly differ from sedentary naïve males; however, NMS-Eex males did produce significantly fewer micturition events in a 30 minute testing period than naïve-Esed males, and NMS and exercise did impact frequency across all groups. **C)** The time between micturition events did not significantly differ among the four groups evaluated, but early exercise exposure had a significant impact on intermicturition intervals overall. Micturition threshold pressure **(D)** and the maximum pressure recorded **(E)** did not differ among the groups. **F)** Pressure amplitude was significantly influenced by an NMS-exercise interaction effect. **G)** NMS-Eex males produced significantly higher void masses compared to naïve-Esed males, and early exercise had a significant impact across all four groups. **H)** Moreover, early exercise significantly influenced void masses per micturition cycle, and NMS-Eex males exhibited significantly higher void mass per cycle than both sedentary groups. **I)** The maximum infused volume of saline into the bladder was significantly dependent on early exercise exposure, but groups did not significantly differ from one another. **J)** The infused volume of saline per cycle was also significantly influenced by early exercise, and NMS-Eex males exhibited significantly greater infused volumes per cycle than both sedentary groups. Brackets indicate significant effect of NMS (§$p < 0.05$), early exercise (†, ††$p < 0.05$, 0.01), or NMS/exercise interaction effect (&p <0.05); two-way ANOVA, *$p < 0.05$ vs. naïve-Esed, ‡$p < 0.05$ vs. NMS-Esed, Bonferroni posttest (n = 3-4).

trended toward fewer voids than naïve males, though groups did not significantly differ from one another (Figure 5.3B). An NMS-exercise interaction effect was only observed for pressure amplitude (Figure 5.3F). Early exercise had the greatest effect on bladder function across all groups for intermicturition intervals, maximum void mass, void mass per micturition cycle, maximum infused saline volume, and infused volume per micturition cycle (Figure 5.3C, G, H, I, and J).

NMS did not induce anxiety-like nor depression-like behaviors, but may have negatively altered reward behavior

Because a significant proportion of CP/CPPS patients also suffer from anxiety and depression [80, 231-233], we investigated murine elevated plus-maze and sucrose preference activity to measure anxiety-like and depression-like behaviors, respectively, before and/or after voluntary wheel running for naïve and NMS males.

The elevated plus-maze is a widely used and validated rodent behavioral assay to assess anxiety-like behavior, typically to evaluate the anti-anxiety effects of pharmacological agents and to characterize brain regions and mechanisms involved in anxiety-related behavior. This test is based on rodents' natural aversion to open and elevated environments and natural spontaneous exploratory behavior to novel environments. We hypothesized sedentary NMS males would spend less time in open arms than sedentary naïve males, and voluntary exercise would prevent or reverse this perceived anxiety-like behavior as measured by longer bouts of exploratory activity in open arms. The time sedentary and baseline NMS males spent in open arms of the plus-maze did not significantly differ from their naïve counterparts (Figure 5.4). Interestingly, early-exercised mice tended to spend less time in open arms than sedentary counterparts (Figure 5.4A) and late-exercised males spent less time in open arms after 8 weeks of voluntary wheel running (Figure 5.4B). This observation following late exercise may be

Figure 5.4 Anxiety-like behavior measured by elevated plus-maze

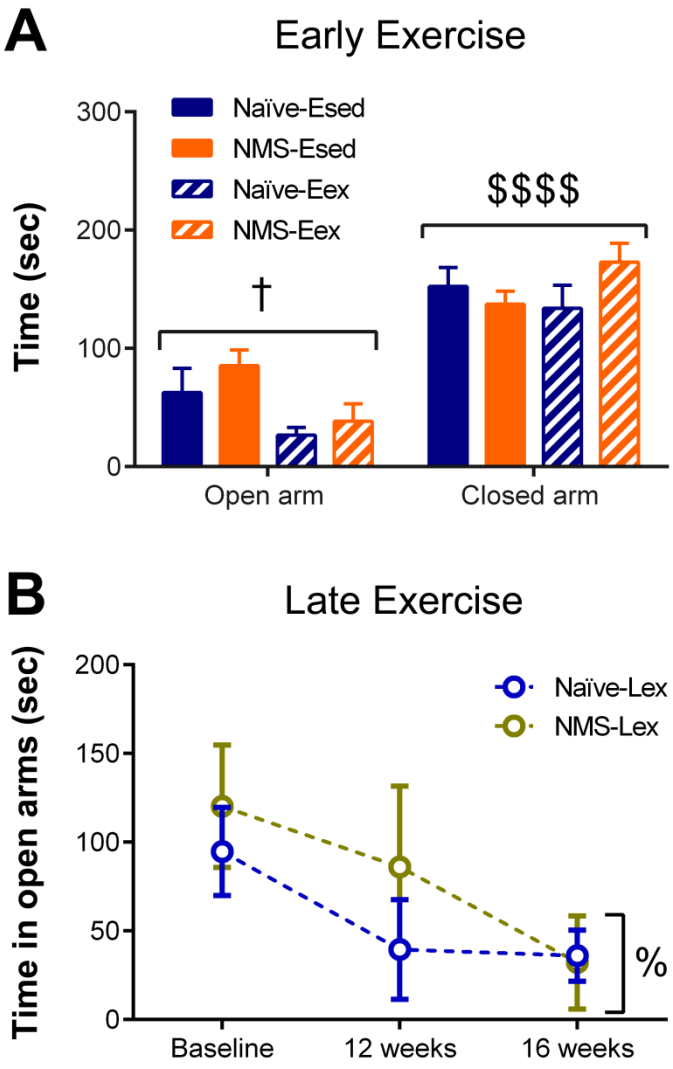

Figure 5.4 Anxiety-like behavior measured by elevated plus-maze. Neither NMS nor voluntary wheel running affected anxiety-like behaviors as measured by elevated plus-maze (EPM). **A)** Each group of mice spent significantly more time in closed arms than exploring open arms. **B)** Similarly, no differences in time spent exploring open arms were observed at any time point between naïve and NMS males exposed to late exercise. **A:** Brackets indicate significant effect of exercise (†$p < 0.05$) or plus-maze arm ($$$$$p < 0.0001$), two-way ANOVA, Bonferroni posttest (n = 7-8). **B:** Bracket indicates significant effect of time (%$p < 0.05$), two-way RM ANOVA, Bonferroni posttest (n = 4-5).

due to a novelty effect; upon introduction to the plus-maze, mice exhibited greater exploratory behavior that tapered off with consecutive testing periods.

Though we did not observe anxiety-like behavior in our NMS males, we were still interested in whether early life stress would influence sucrose preference as a measure of anhedonia-like behavior. We hypothesized NMS mice would consume less sucrose solution than naïve controls when presented with both standard drinking water and a 1% sucrose solution and exercise would prevent or reverse anhedonia-like behavior. NMS did not, however, affect sedentary animals' preference for sucrose (Figure 5.5). Early exercise did not significantly impact the percent of sucrose consumed for neither naïve nor NMS males, but naïve-Eex males consumed significantly more sucrose solution by volume than their sedentary controls (Figure 5.5A and B, respectively). Naïve-Lex males exhibited a decrease in sucrose preference compared to naïve-Lsed controls, and NMS-Lex males showed a significantly higher preference for sucrose than naïve-Lex males (Figure 5.5C). Late-exercised groups did not significantly differ in their percent sucrose preference when compared to one another or their respective baselines at 8 weeks of age when analyzed by two-way repeated measures ANOVA; however, when analyzed with standard two-way ANOVA with Bonferroni posttest, there was a significant effect of NMS on sucrose preference (§$p < 0.05$) (Figure 5.5D).

While decreases in sucrose consumption can be interpreted as depression-like behavior, sucrose preference can also indicate an alteration in overall reward behavior. Additionally, exercise is considered a natural reward for rodents, and rodents will initiate and sustain running activity when presented with uninhibited access to a free running wheel. Interestingly, we observed an effect of NMS exposure on running wheel activity for both early and late exercised mice (Figure 5.6). Early-exercised NMS male pairs ran shorter average distances per day than naïve counterparts (Figure 5.6A). Similarly, singly housed late-exercised NMS males ran shorter average distances per day at the end of the six-week study than naïve-Lex males (Figure 5.6B).

Figure 5.5 Sucrose preference following early and late exercise paradigms

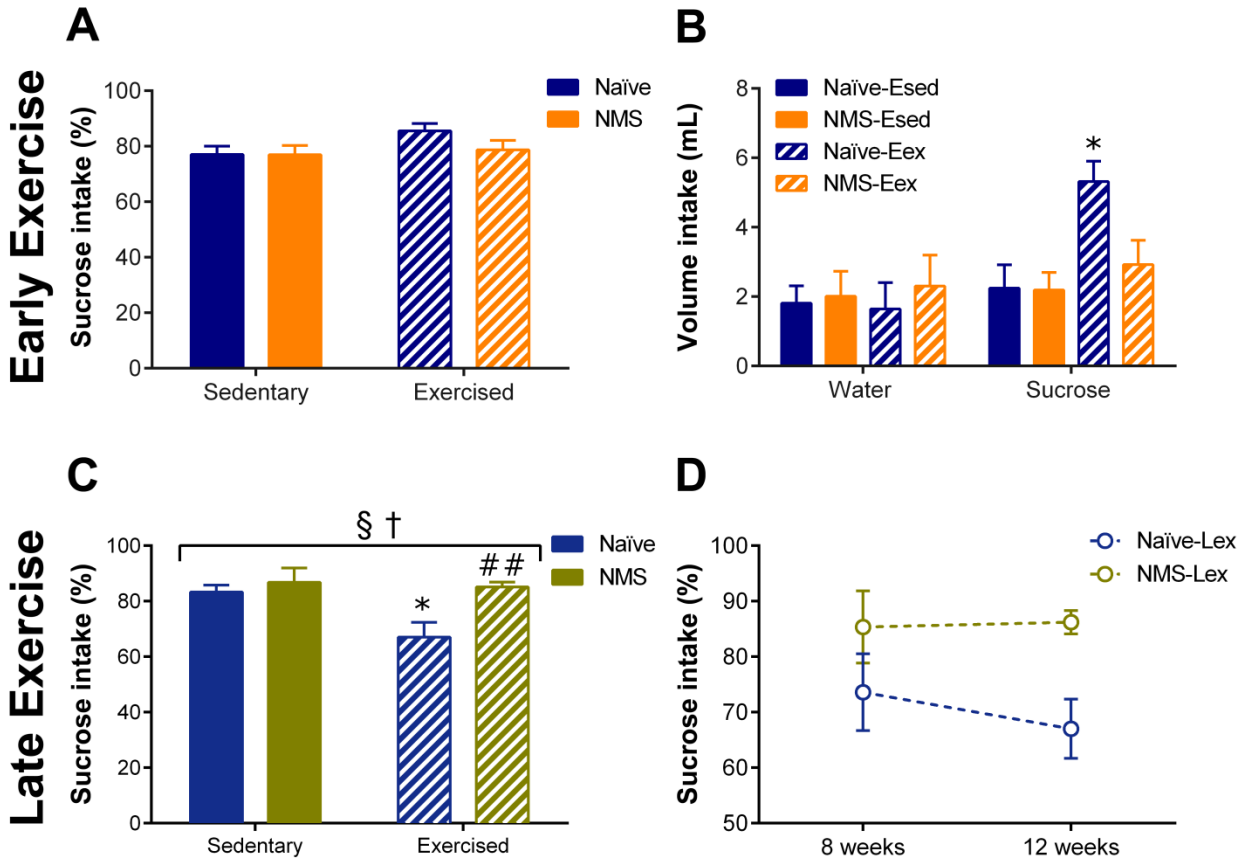

Figure 5.5 Sucrose preference following early and late exercise paradigms. NMS did not affect sedentary animals' preference for sucrose. Early exercise did not significantly impact the percent of sucrose consumed for naïve nor NMS males **(A)**, but naïve-Eex males consumed significantly more sucrose solution by volume than their sedentary controls **(B)**. **C)** Naïve-Lex males exhibited a decrease in sucrose preference compared to naïve-Lsed controls and NMS-Lex males showed a significantly higher preference for sucrose than naïve-Lex males. **D)** Late-exercised groups did not significantly differ in their percent sucrose preference when compared to one another or their respective baselines at 8 weeks of age. Brackets indicate significant effect of NMS (§$p < 0.05$) and exercise (†$p < 0.05$); two-way ANOVA, *$p < 0.05$ vs. sedentary naïve, ##$p < 0.01$ vs. exercised naïve, Bonferroni posttest (n = 5-27).

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Figure 5.6 NMS males ran shorter average distances than naïve males

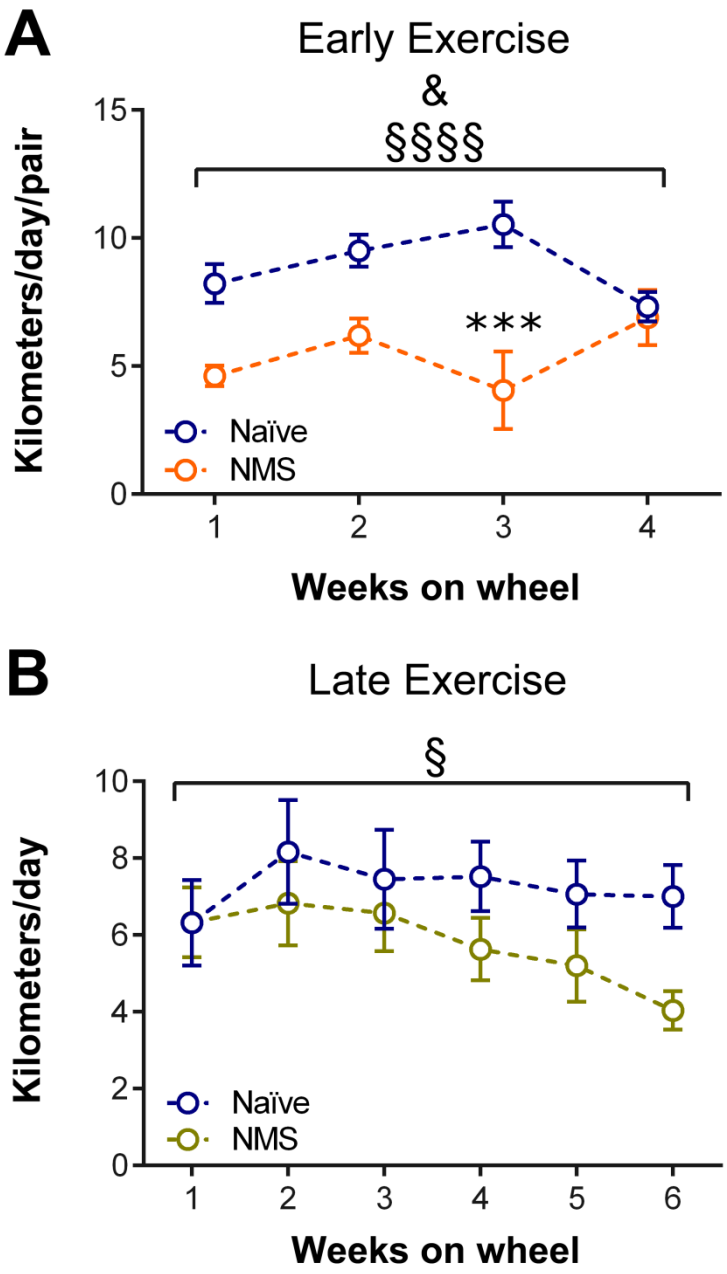

Figure 5.6 NMS males ran shorter average distances than naïve males. **A)** NMS status and an NMS-exercise interaction effect had a significant impact on running wheel activity: early-exercised NMS pairs ran shorter average distances than naïve pairs. NMS pairs ran significantly shorter distances after 3 weeks on wheels, but matched naïve distances after 4 weeks of wheel running. **B)** NMS also affected running wheel activity for late-exercised males. Naïve and NMS male ran similar average distances during the first wheel of exercise, but NMS males ran decreasingly shorter distances over the following 5 weeks of late exercise. Brackets indicate significant effect of NMS (§, §§§§$p < 0.05$, 0.0001) and/or an NMS-exercise interaction (&$p < 0.05$), two-way ANOVA, ***$p < 0.001$ vs. Naïve-Eex, Bonferroni posttest (n = 3-8).

These data, sucrose preference and running distance, may indicate a perturbation in reward circuitry. It has been argued regular physical activity is the natural state for both rodents and humans, so when observing the data through this lens, we see an indication of NMS blunting natural pleasure-seeking behavior following exercise that was not observed in sedentary controls.

Exercise prevented and reversed NMS-induced mast cell degranulation in urogenital tissues

Cryosections of bladder and prostate tissues were stained using toluidine blue-O to stain mast cells. Intact and degranulated mast cells were quantified and the degranulated percentage was calculated. Consistent with our previous results [137], sedentary NMS tissues exhibited increased mast cell degranulation compared to sedentary naïve controls (Figure 5.7). Early and late exercise decreased NMS-induced mast cell degranulation. These data suggest voluntary running wheel activity is able to prevent and reverse increases in mast cell degranulation associated with NMS.

NMS reduced HPA axis output

To examine central gene expression changes, hypothalamic, hippocampal, and amygdalar issues were collected from early and late sedentary and exercised males for RT-PCR associated with HPA axis action and regulation. Additionally, we utilized a rodent corticosterone (CORT) ELISA kit to measure concentrations of circulating serum CORT as a downstream measure of HPA axis output.

We observed early exercise had a significant impact on hypothalamic increases in CRF_2 gene expression (Figure 5.8A), as well as hippocampal BDNF (Figure 5.8B). Groups did not significantly differ from one another when evaluated for expression of CRF, CRF_1, Ucn2 (hypothalamus only), GR, or MR in both hypothalamic and hippocampal tissues of males

Figure 5.7 Exercise normalized NMS-induced mast cell degranulation in urogenital tissues

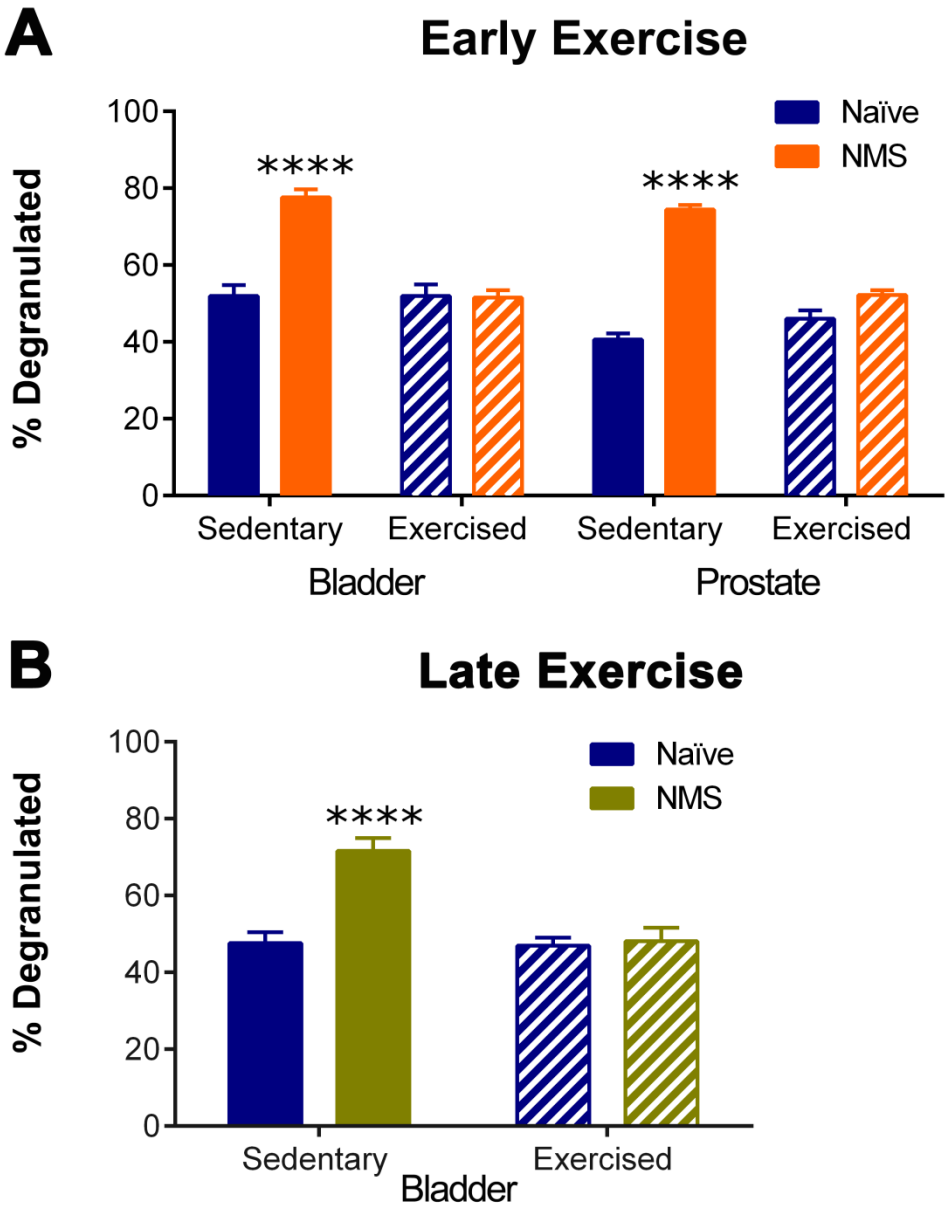

Figure 5.7 Exercise normalized NMS-induced mast cell degranulation in urogenital tissues. Acidified toluidine blue was used to visualize tryptase granules and calculate the percentage of activated/degranulated mast cells in cryostat sections of bladder and prostate tissues. **A)** Bladders and prostates from sedentary mice subjected to NMS exhibited significantly higher percentages of degranulated mast cells compared to sedentary naïve males, and normalized to naïve-Esed levels following early exercise. **B)** NMS-Lsed bladders exhibited significantly higher percentages of degranulated mast cells compared to naïve-Lsed, and were normalized to naïve levels following late exercise. Two-way ANOVA; ****$p < 0.0001$ vs. sedentary naïve, Bonferroni posttest (n = 2-4).

Figure 5.8 Gene expression changes within limbic structures associated with the HPA axis following exercise

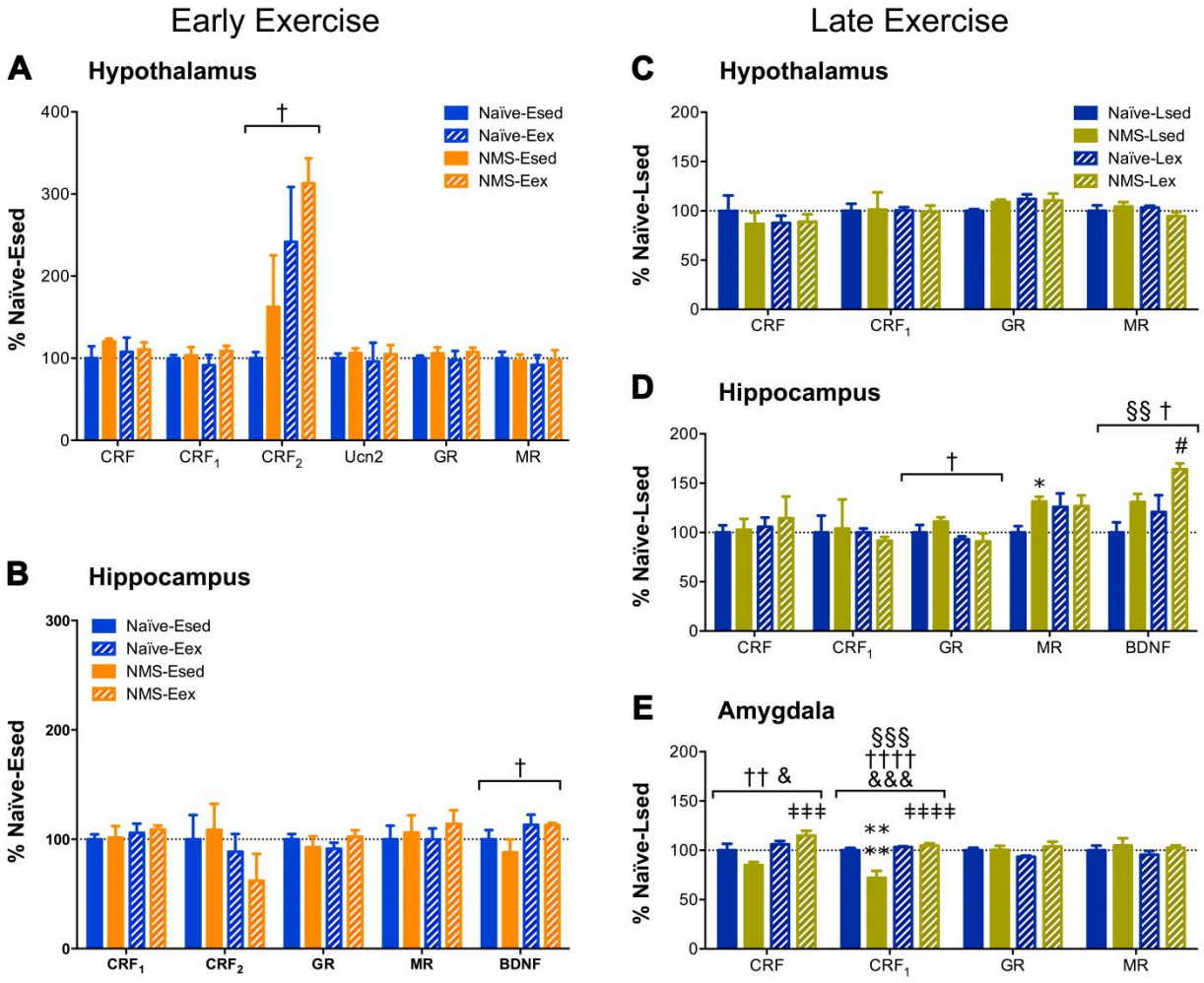

Figure 5.8 Gene expression changes within limbic structures associated with the HPA axis following exercise. Early exercise had a significant impact on hypothalamic CRF_2 gene expression **(A)**, as well as hippocampal BDNF **(B)**. Late exercise had a significant effect on hippocampal GR and BDNF mRNA levels **(D)**, as well as amygdalar CRF and CRF_1 **(E)**. An effect of NMS was only observed for hippocampal BDNF **(D)** and amygdalar CRF_1 **(E)**. **D)** NMS-Lsed hippocampal MR mRNA was significantly increased compared to naïve-Lsed controls. NMS-Lex hippocampal BDNF mRNA was also significantly increased compared to naïve-Lex. **E)** NMS-Lex compared to NMS-Lex displayed greater mRNA levels of CRF and CRF_1 in amygdala tissue. Sedentary NMS mRNA levels only differed from sedentary naïve levels when comparing amygdala CRF_1; NMS-Lsed levels were significantly lower than naïve-Lsed. An NMS/late exercise interaction effect was observed for amygdalar CRF and CRF_1 mRNA levels. Brackets indicate significant effect of NMS (§§, §§§p < 0.01, 0.001), exercise (†, ††, ††††p < 0.05, 0.01, 0.0001), and/or an NMS-exercise interaction effect (&, &&&p < 0.05, 0.001); two-way ANOVA, *p < 0.05 vs. sedentary naïve, #p < 0.05 vs. exercised naïve, ǂǂǂ, ǂǂǂǂp < 0.001, 0.0001 vs. sedentary NMS, Bonferroni posttest (n = 3-8).

exposed to early sedentary or exercise conditions (Figure 5.8A and B). HPA axis regulation was not altered dramatically, but we did observe lower concentrations of serum CORT for NMS-Esed males compared to naïve-Esed (Figure 5.9A), though we also saw early exercise lowered circulating CORT for both naïve- and NMS-Eex males compared to naïve-Esed males. Overall, NMS and exercise had significant impacts on serum CORT concentrations.

Late exercise had a significant effect on hippocampal GR and BDNF mRNA levels (Figure 5.8D), as well as amygdalar CRF and CRF_1 (Figure 5.8E). Additionally, an effect of NMS was only observed for hippocampal BDNF (Figure 5.8D) and amygdalar CRF_1 (Figure 5.8E), and an NMS/late exercise interaction effect was observed for amygdalar CRF and CRF_1 mRNA levels (Figure 5.8E). Neither NMS nor late exercise effected hypothalamic genes. NMS-Lsed hippocampal MR mRNA were significantly increased compared to naïve-Lsed controls, and NMS-Lex hippocampal BDNF mRNA was also significantly increased compared to naïve-Lex (Figure 5.8D). NMS-Lex compared to NMS-Lex displayed greater mRNA levels of CRF and CRF_1 in amygdala tissue. Sedentary NMS mRNA levels only differed from sedentary naïve levels when comparing amygdala CRF_1; NMS-Lsed levels were significantly lower than naïve-Lsed. Though gene expression changes were variable, serum CORT concentrations for sedentary NMS males were statistically lower than sedentary naïve controls, consistent with our previous results for the early exercise and WAS studies; however, late-exercised NMS males displayed a greater range of concentrations, contributing to the variability and large error bars (Figure 5.9B).

Figure 5.9 Serum corticosterone concentrations

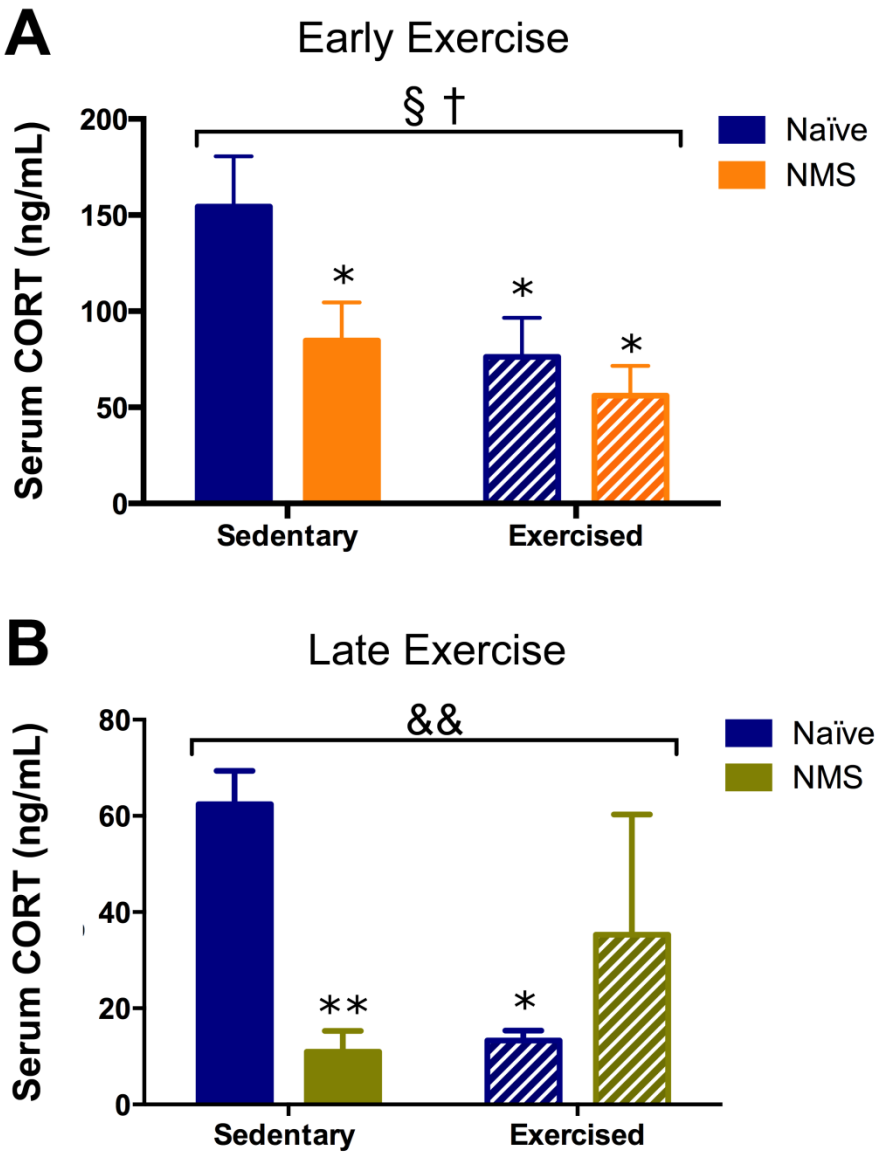

Figure 5.9 Serum corticosterone concentrations. **A)** NMS-Esed, naïve-Eex, and NMS-Eex mice exhibited decreasingly lower concentrations of serum corticosterone (CORT) compared to sedentary naïve controls. **B)** Sedentary NMS and late-exercised naïve males displayed lower serum CORT concentrations compared to sedentary naïve mice; however, late-exercised NMS males displayed a range of serum CORT concentrations that did not significantly differ from the other groups. Brackets indicate an effect of NMS (§$p < 0.05$), exercise (†$p < 0.05$), or NMS/exercise interaction (&&$p < 0.01$); two-way ANOVA, *, **$p < 0.05$ vs. sedentary naïve, Bonferroni posttest (**A:** n = 10; **B:** n = 3-7).

Chapter VI: Discussion

Neonatal maternal separation increases susceptibility to experimental colitis and acute stress exposure in male mice

Exposure to early life stress or trauma is a significant risk factor for developing several chronic pain syndromes, including IBS [19-21]. NMS has long been used in rodents to model both psychological disturbances and heightened colorectal sensitivity commonly experienced by IBS patients. Here, we performed a prolonged NMS paradigm in male mice and provided evidence of increased susceptibility of the distal colon to experimental colitis using TNBS and acute stress exposure, with accompanying alterations in pro-inflammatory gene/CRF receptor expression.

Rodent models of NMS [175, 234-236] and stress-sensitive strains of rodents [237] have been shown to display behaviors indicative of increased anxiety and depression. We previously reported a significant decrease in baseline anxiety-like behavior and thermal and mechanical thresholds in the hind paw of female NMS mice at baseline [136]. In the current study, male NMS mice displayed no significant change in anxiety-like behaviors, yet did have significantly shorter thermal withdrawal latencies at baseline. Further exposure to stress, in the form of CRD, significantly increased anxiety-like behaviors, similar to the increase observed in female NMS mice following vaginal balloon distension [136]. Interestingly, mechanical withdrawal thresholds were significantly lower in NMS than naïve mice, post-CRD; however, thermal withdrawal latencies were no longer significantly shorter in NMS mice. Exposure to VBD maintained decreased hind paw thresholds in our previous study of female NMS mice [136] and sex differences in behavioral outcomes following NMS have been previously reported [238, 239] and likely contribute to the more robust response in the female NMS mice, compared to the male.

Increased colorectal sensitivity is a commonly reported outcome of NMS and other manipulations of the stress-response system. High-anxiety strains of rats demonstrate

significantly greater colorectal sensitivity than non-anxious strains [240-242]. Several studies of NMS in rats have reported increased colorectal sensitivity measured as either increased VMR [179, 243, 244] or visualized abdominal withdrawal reflexes [185, 239, 245, 246] during CRD. In the current study, as well as in our previous study of female NMS mice [247], we did not observe any evidence of colorectal sensitivity during CRD in NMS mice at baseline. This is in contrast to the significant perigenital mechanical sensitivity exhibited by male NMS mice [248] and vaginal [136] and bladder [247] hypersensitivity exhibited by female NMS mice, suggesting that the NMS paradigm employed in our studies selectively sensitizes the urogenital system at baseline. To our knowledge, only one previous study has investigated colorectal sensitivity in NMS mice and reported a significant increase in VMR during CRD [181]. This study used BALB/c mice, which have been shown to be a higher anxiety strain than C57Bl/6 mice [249], and incorporated NMS from P1-14 with concurrent maternal stress (forced swimming or restraint) during the separation period.

Evidence of increased susceptibility to experimental colitis was observed in NMS mice, despite the lack of increased colorectal sensitivity four days after treatment with either 2 or 5mg TNBS. Previous studies of NMS in mice reported increased MPO and cytokine production, as well as elevated histological scores [250-252]. Here, we demonstrated a greater loss of body weight and decreased survival in NMS mice following 5mg TNBS. Colonic MPO activity was continuing to increase at 4d post-TNBS in NMS mice, while MPO activity had decreased from its peak at 1d post-TNBS in naïve mice, as has been shown previously [253], suggesting an exaggerated inflammatory response in NMS mice. Cytokine and growth factor production have also been shown to simultaneously increase in rodent models of colitis [254] and ulcerative colitis patients have an increased production of pro-inflammatory cytokines [255], as well as greater expression of neurotrophic receptors in peripheral nerve endings innervating the diseased colon [256]. Naïve mice treated with 5mg TNBS showed a trend toward increased

colonic IL-6 mRNA levels, which was not apparent in NMS colon. Two growth factors, nerve growth factor (NGF) and artemin, have been shown to dramatically increase following acute colitis in rodents [257-259]. Here we observed a slight, but not significant, increase in artemin mRNA levels in naïve mice following 5mg TNBS and NMS colon actually showed a significant decrease in artemin mRNA levels compared to naïve colon. Colonic NGF mRNA was not significantly altered by 5mg TNBS in either naïve or NMS mice (data not shown).

Symptom onset or exacerbation is often triggered by stress in patients suffering from IBS [12]. Clinically, peripheral administration of CRF has been shown to reduce the threshold to CRD and increase rectal compliance in human subjects [260]. Likewise, administration of CRF_1 antagonist produced inhibitory effects within the emotional-arousal circuit of female IBS patients that were anticipating a painful stimulus [24]. A single exposure to WAS has previously been shown to increase VMR in NMS rats, but not in naïve rats [31, 32, 179], whereas repeated exposure to WAS has been shown to significantly increase colorectal sensitivity in non-NMS rats [261, 262]. In the current study, colorectal sensitivity in NMS mice was only increased by a single exposure to WAS and not following chronic exposure. The VMR of naïve mice was completely unaffected by either WAS treatment. Considering the paucity of studies investigating the impact of NMS or WAS on colorectal sensitivity in mice, it is possible that these stressors do not have the same impact across species. This theory is supported by our previous studies showing an impact of NMS on urogenital sensitivity in mice [136, 247, 248], which has largely not been observed in rat models of NMS.

Rodents exposed to NMS demonstrate increased growth factor and cytokine expression, including NGF, IL-6, IL-1β, IL-2, IL-4, IL-10, and interferon (IFN)-γ [26-29], as well as infiltration of mast cells [30-32], in the distal colon, all of which can sensitize peripheral nociceptors and enhance visceral perception [26-29, 33]. Mast cell infiltration and hypertrophy of sensory innervation has also been reported in biopsies from patients with IBS [34-36]. A single exposure

to WAS elicited a significant increase in IL-6 mRNA levels in naïve colon, confirming that, despite the lack of physiological evidence, 1d WAS exposure did indeed significantly impact naïve colon. The mRNA levels of IL-6 in NMS mice trended toward a significant increase; however, the blunted effect of WAS on increasing IL-6 expression in NMS colon further suggests that neuroimmune responses to stress exposure are imbalanced following NMS exposure in mice.

A potential role for CRF in mediating comorbidity between psychological and chronic pain disorders has been investigated in both clinical and preclinical settings. Over-activity of central CRF_1 signaling has been proposed to contribute towards comorbid anxiety/depression in female diarrhea-predominant IBS patients [23]. Associations between anxiety and voiding disorders have also been reported [90] and interstitial cystitis (IC) patients with fibromyalgia, chronic fatigue syndrome, or rheumatoid arthritis had higher mean afternoon cortisol levels and increased pain during bladder filling than IC patients with no additional diagnoses [91]. In a study using repeated WAS exposure to induce colorectal sensitivity in non-NMS rats, treatment with a CRF_1 antagonist treatment prior to, but not following, WAS prevented an increase in VMR [263]. However, treatment with astressin, a non-selective CRF_1/ CRF_2 antagonist, only reduced the effect of WAS, suggesting that activation of CRF_2 mediates colonic hypoalgesia. This observation is supported by studies showing that activation of CRF_2 decreases CRF_1-driven colorectal hypersensitivity [264, 265], c-Fos induction in the colonic myenteric ganglia, and increased colonic motility [266]. In the current study, protein levels of CRF_2 were increased in NMS colon at baseline, similar to previous studies investigating mRNA levels in both male NMS rats [113] and female NMS mice [136]. The protein levels of CRF_2 were also increased in naïve colon following a single exposure to WAS. Urocortin signaling through CRF_2 has been shown to increase IL-6 expression/release in cardiomyocytes and aortic smooth muscle cells [267, 268], and could be playing a similar mechanistic role here. The baseline increase in CRF_2 protein in

NMS colon may have primed the response to WAS, thereby increasing colorectal sensitivity, compared to naïve mice. Further investigation will be required to understand how the CRF receptors mediate colorectal sensitivity in mice and how stress at both early and late stages of life will impact their expression and activation patterns.

Conclusions

We have tested the hypothesis that early life stress in male mice increases susceptibility to experimental colitis and adult stress exposure. Mice exposed to NMS show increased thermal hind paw sensitivity at baseline with an increase in anxiety-like behaviors and mechanical hind paw sensitivity following exposure to CRD. Intracolonic instillation of TNBS induces a dose-dependent loss in body weight and decreased survival rate in NMS mice. A single exposure to WAS, but not a 7-day repeated exposure, increased the VMR during CRD of NMS mice, and revealed evidence of improper neuroimmune response within the colon. Taken together, these results suggest that NMS in mice disrupts inflammatory- and stress-induced gene expression in the colon, potentially contributing towards an exaggerated response to specific stressors later in life.

Water avoidance stress did not compound phenotypes associated with neonatal maternal separation in male mice

NIH designated category III chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) and interstitial cystitis/painful bladder syndrome (IC/PBS) share overlapping symptomology and are frequently co-diagnosed [63, 65]. For many of these patients, stress often triggers or exacerbates symptoms and share a history of early life stress [232, 269]. Early life stress or experiences of adverse childhood events have been associated with improper functioning of the hypothalamic-pituitary-adrenal (HPA) axis and can serve as risk factors for functional pain disorders in adulthood [21, 105, 232, 269]. Here we have provided the first evidence of early life

stress (NMS)-induced perigenital mechanical sensitivity of male mice that was not, however, exacerbated by acute adult stress, in the form of water avoidance stress (WAS). Experience of acute adult stress did not increase urine output of NMS-exposed mice, but did enhance neuroimmune profiles in both bladder and prostate of naïve mice, though NMS had a more significant impact on mast cell degranulation. These data suggest that early life stress contributes to a painful phenotype that is variably affected by acute stress in adulthood.

CP/CPPS and IC/PBS are characterized by chronic, idiopathic pelviperineal referable pain with or without urinary symptoms such as urgency, frequency, and nocturia [39, 65]. It has been estimated that as high as 17% of CP/CPPS patients suffer from comorbid IC/PBS [63]. These patients frequently report the onset or worsening of symptoms during periods of heightened stress [11, 81, 99, 100]. Additionally, a proportion of these individuals also report a history of adverse childhood events, and exposure to early life stress or trauma has been shown to be a significant risk factor developing HPA axis abnormalities and associated chronic pain syndromes [21, 105, 269]. NMS in rodents has been used for several decades as a model of early life stress that significantly impacts the functioning of the HPA axis [168-171]. We have repurposed this well-validated model for colonic sensitivity to investigate perigenital sensitivity in male mice as a novel model for CP/CPPS with comorbid IC/PBS and mood disturbances. In a previous study, we reported a significant decrease in mechanical withdrawal thresholds in the perigenital region of male NMS mice at baseline [137]. In the current study, male NMS mice similarly displayed significantly lower withdrawal thresholds compared to naïve controls at baseline, but this perigenital allodynia was not exacerbated by acute WAS exposure. Furthermore, WAS did not enhance micturition output observed in NMS males compared to naïve controls. We also observed NMS mice to display increased gastrointestinal output compared to naïve, overall and at baseline. A correlation between gastrointestinal and total urinary output was only observed for naïve mice at baseline and 8d post-WAS.

As CP/CPPS and IC/PBS patients commonly suffer from depression [79, 82-84, 87, 88], we were interested in investigating sucrose preference as a measure of anhedonia-like behavior. We did not, however, observe any differences in sucrose preference between NMS and naïve mice. We also hypothesized WAS exposure would reduce sucrose consumption in NMS males. We did not see a decrease in NMS sucrose preference; in fact, both NMS and naïve males increased their consumption from baseline following WAS exposure. Finding no differences at baseline may be due to strain choice; C57BL/6 mice have been reported to be stress-resilient to social-defeat, repeated footshock, and cat urine exposure [270-273]. For future studies it may be prudent to select a less resilient strain. Another member of the lab has had preliminary success inducing behavioral measurements of anhedonia with concomitant hypersensitivity using repeated footshock in A/J mice (The Jackson Laboratory). A/J mice have been shown to exhibit more anxiety-like and depression-like behaviors [274], but is an inbred species more commonly used to model cancer and carcinogenic testing.

Increased mast cell infiltration and degranulation has been implicated in CP/CPPS as well as IC/PBS [43, 54, 71, 72, 74, 94, 148-156, 158, 160, 161, 163-165]. Previous work from our lab has shown neonatal maternal separation to influence mast cell degranulation in male C57Bl/6 mice [137]. Interestingly, WAS resulted in increased mast cell degranulation in only the prostates, not bladders, of naïve males compared to unstressed controls, though NMS had a far more significant impact on mast cell degranulation in both bladder and prostate tissues. Tissue-specific differences were also observed for inflammatory gene expression changes in response to NMS and/or WAS exposure. In bladder, increases in NGF expression were significantly affected by NMS, as well as an NMS/WAS interaction effect. NMS-WAS males displayed the greatest increase of NGF mRNA levels; significantly greater than both naïve and NMS controls. An NMS/WAS interaction effect also had a significant impact on increased MCP-1 mRNA levels. Similar to bladder values, prostatic IL-6 and IL-10 were not statistically altered by NMS or WAS exposure. We had expected both IL-6 and IL-10 to be elevated based on the findings of other

investigators [58, 59, 74]. Expression of SCF was not altered in bladders across all four groups; however, in the prostates, decreases in SCF expression were attributed to NMS and NMS/WAS interaction effects. Only NMS controls expressed significantly less SCF mRNA than naïve controls. WAS had a significant impact on increases in MCP-1.

Increases in NGF are consistent with other studies and clinical presentation, though investigating levels of mast cell tryptase should also be a priority for future studies; tryptase has been shown to be elevated in IC/PBS urine [74, 163-165] and CP/CPPS prostatic secretions [43]. Elevated mast cell tryptase in CP/CPPS suggests mast cells are present in the prostate and in a state of activation [43]. Increased NGF expression has been previously noted in IC/PBS urine [74, 163-165], CP/CPPS prostatic secretions, and EAP models of prostatitis [57, 166]. Mast cell tryptase and NGF have been considered the most promising biomarkers of CP/CPPS, and NGF's prominent role in IC/PBS further strengthens the hypothesis that CP/CPPS and IC/PBS are different manifestations of the same underlying disorder. NGF can be released from mast cells, as well as contribute to degranulation [43]. This is significant because there is evidence that nerves and mast cells may communicate in an NGF-dependent manner [275]. NGF is a potential mediator of peripheral sensitization mechanisms underlying CP/CPPS [43, 276] because of its role in generation and potentiation of pain following tissue injury and inflammation [277]. NGF has become a therapeutic target, yielding marginal success; therapeutic inhibition of NGF decreased pain-like behavior responses in a number of animal models of visceral pain [278], as well as IC/PBS in humans [279].

To determine the impact of NMS and WAS on gene expression within central structures involved in the regulation and output of the HPA axis, mRNA levels of corticotropin releasing factor (CRF), urocortin 2 (Ucn2), CRF receptor 1 (CRF_1), CRF_2, glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) in hypothalamus, amygdala, and hippocampus of mice were determined by RT-PCR. WAS exposure had a significant impact on decreases in CRF,

Ucn2, GR, and MR mRNA expression in hippocampi. CRF and MR were particularly low for both naïve and NMS WAS-exposed groups, whereas only naïve-WAS males displayed a statistically lower level of Ucn2 mRNA than controls. In contrast, WAS had a significant impact on increases in CRF, CRF_1, GR, and MR mRNA levels in amygdalae. WAS-exposed naïve and NMS groups showed significantly higher levels of CRF_1, GR, and MR mRNA compared to their baseline counterparts. A similar trend was observed for CRF_1 mRNA levels in hippocampi. Increases in GR were statistically attributed to an NMS/WAS interaction effect, and MR to both WAS and an interaction effect. Additionally, NMS controls and naïve WAS-exposed males exhibited similar increases in MR mRNA expression. When these data are taken together with the changes in peripheral CRF_1 and lower serum CORT concentrations, we conclude our NMS males exhibit something resembling hypocortisolism, similar to studies in children exposed to severe deprivation, neglect, or abuse reporting lower baseline levels of glucocorticoids [112, 125], and WAS induces changes to gene expression that may decrease HPA axis output. In human studies, it has been hypothesized that lower basal glucocorticoid levels may be due to a downregulation of the HPA axis at the level of the pituitary in response to chronic drive of CRF from the hypothalamus [126], or target tissue hypersensitivity to glucocorticoids [127]. Stress exposure typically promotes CRF release in the central amygdale [130]. Chronic glucocorticoid exposure increases expression of CRF mRNA [131, 132], suggesting a stress sensitization that may be involved in the development of stress-related pathologies [120]. Our reported mRNA levels, however, were not significantly different for NMS amygdale, and we saw only marginal increases in CRF expression for both WAS groups compared to naïve controls.

Conclusions

We have tested the hypothesis that early life stress in male mice increases perigenital hypersensitivity, micturition pattern, and depression-like behavior, as well as possible exacerbation by acute adult stress. Mice exposed to NMS show increased perigenital

mechanical sensitivity at baseline, but did not worsen following WAS. Baseline micturition patterning assays showed NMS males producing a greater number of void and greater urine output; these measurements were dramatically lower after WAS, but this may in part be due to a loss of novelty/exploratory behavior during the micturition testing period. We also failed to observe any indications of altered mood behavior. Most promising were data indicating enhanced neuroimmune profiles, central gene expression changes, and peripheral HPA axis output measures following both NMS and WAS exposure. Though our data did not mirror the majority of previous studies, taken together, these results suggest that NMS in male mice induces perigenital hypersensitivity, enhances mast cell degranulation and inflammatory gene expression, and results in decreased HPA axis output; though WAS did not exacerbate pain-like behaviors, WAS did impact mast cell degranulation and gene expression changes associated with stress sensitization.

Exercise prevented and reversed prominent neonatal maternal separation-induced perigenital hypersensitivity and urogenital tissue mast cell degranulation

Exercise and regular physical activity can significantly improve symptom severity in patients with IBS [193, 195-199], fibromyalgia [200-202], and depression and/or anxiety [203-205]. Voluntary physical activity can favorably influence brain plasticity by facilitating neurogenerative, neuroadaptive, and neuroprotective processes [187]. Chronic voluntary physical activity also attenuates neural responses to stress in brain circuits responsible for regulating peripheral sympathetic activity; mitigating several harmful consequences of acute stress exposure [187]. We first observed NMS-induced perigenital hypersensitivity, consistent with our previous studies [137], to be prevented or reversed by running wheel activity. Here we provide the first evidence of the efficacy of voluntary wheel running as a potential therapeutic intervention to attenuate the painful phenotype induced by early life stress exposure (NMS) as well as alterations to micturition patterning, mast cell degranulation, and HPA axis output. We

are confident these improvements in perigenital sensitivity and mast cell degranulation are attributable to physical activity rather than enhanced environmental enrichment of the running wheel; Nyhuis *et al*. previously reported that only exercised rats showed rapid glucocorticoid habituation to repeated audiogenic stress [280]. For future studies, however, it may be advantageous to include locked wheels in sedentary males' home cages to control for the added enrichment.

Increased urinary urgency and frequency are characteristic symptoms of IC/PBS and, in some cases, CP/CPPS. Similar to our previous results, NMS males exhibited increased void frequency and urine output. Micturition patterns were returned to naïve levels following both early exercise and late exercise. However, both naïve and NMS void measurements dropped sharply when reassessed. This noted change in behavior may be due to a lack of novelty; when mice were reintroduced to the testing setup, exploratory behavior decreased. To more accurately measure bladder function, a catheter was implanted into the dome of the bladder and subcutaneously tunneled and exteriorized at the base of the neck for the purposes of performing awake, filling cystometry. Schwartz *et al*. previously reported increased urinary voiding frequency for a murine model of chronic prostatitis by zymosan injection to the prostate [58]. In our NMS model, however, we observed a trend toward fewer void events, as well as greater threshold pressures, greater pressure amplitudes, and greater volumes and masses of void events, especially following early exercise. Additional studies need to be performed to increase statistical strength and determine how cystometric analysis differ from behavioral micturition testing. It is likely that voluntary voiding, particularly in a prey species, is regulated by more operant mechanisms than cystometric measurements can provide. However, altering our surgical techniques to increase reproducibility and diminish external input, such as stabilizing the catheter at the base of the neck to decrease movement of the catheter at the bladder and providing a sound-proof test space, will provide more consistent data.

In rodents, exercise has been shown to attenuate both anxiety- and depression-related behaviors in NMS rats by normalizing gene expression changes within limbic structures regulating HPA axis output [188, 189]. Chronic wheel running in rats prevents behavioral consequences of uncontrollable stress, including features of depression and anxiety [187, 190-192]. Free access to running wheels also normalized hippocampal GR and BDNF mRNA levels in NMS rats [188]. BDNF has been reported to be decreased when glucocorticoids are elevated during stress [281]. In a separate study, exercised NMS rats exhibited a significant decrease in depressive behavior compared to sedentary counterparts [189]. We, however, did not observe anxiety-like nor depression-like behaviors. Again, this may be due to the strain choice. However, improvements can be made to EPM and sucrose preference protocol. Arabo *et al*. concluded from their studies that rodents do not immediately behave according to the approach-avoidance conflict when forcibly exposed to the maze and the first 2 minutes of a 5 minute EPM test is not accurate anxiety-like behavior. Another type of maze, such as a zero maze, could also be employed. As for sucrose preference, our mice were acclimated for 24 hours then tested for sucrose preference for 24 hours during our exercise studies (this is not the case for our WAS studies). Establishing a baseline of sucrose preference for a minimum of 4 days prior to sucrose preference data collection, may give us a clearer picture of depression-like behavior, if it is present. Moreover, collecting sucrose data for 2 days, rather than 1, and switching the position of the bottles can control for a potential side preference of the mouse.

Activation of CRF_1 and CRF_2 work in opposition of one another, driving and dampening HPA output, respectively [114-117]. We had expected NMS to decrease HPA axis output, similar to the mRNA levels observed for our NMS controls in our WAS study. We did not observe many changes in gene expression attributed to NMS, but we did observe that early exercise had a significant impact on hypothalamic increases in CRF_2 gene expression, as well as hippocampal BDNF. HPA axis regulation was not altered dramatically, but we did observe

lower concentrations of serum CORT for NMS-Esed males compared to naïve-Esed, consistent with our previous studies, though we also saw early exercise lowered circulating CORT for both naïve- and NMS-Eex males compared to naïve-Esed males. Overall, NMS and exercise had significant impacts on serum CORT concentrations.

Late exercise had a significant effect on hippocampal GR and BDNF mRNA levels, as well as amygdalar CRF and CRF_1. Additionally, an effect of NMS was only observed for hippocampal BDNF and amygdalar CRF_1, and an NMS/late exercise interaction effect was observed for amygdalar CRF and CRF_1 mRNA levels. Neither NMS nor late exercise affected hypothalamic gene expression levels. NMS-Lsed hippocampal MR mRNA were significantly increased compared to naïve-Lsed controls, and NMS-Lex hippocampal BDNF mRNA was also significantly increased compared to naïve-Lex. NMS-Lex compared to NMS-Lex displayed greater mRNA levels of CRF and CRF_1 in amygdala tissue. Sedentary NMS mRNA levels only differed from sedentary naïve levels when comparing amygdala CRF_1; NMS-Lsed levels were significantly lower than naïve-Lsed. Though gene expression changes were variable, serum CORT concentrations for sedentary NMS males were statistically lower than sedentary naïve controls, consistent with our previous results for the early exercise and WAS studies; however, late-exercised NMS males displayed a greater range of concentrations, contributing to the variability and large error bars.

Conclusions

Here we provided evidence early and late exercise can prevent and reverse NMS-induced perigenital allodynia, as well as normalize mast cell degranulation in urogenital tissues. We did not observe evidence of comorbid mood disorders, specifically anxiety-like and depression-like behaviors. NMS did not significantly impact central gene expression in CRF-responsive brain regions, but serum CORT concentrations are consistent with our previous results of NMS inducing a state of lower basal serum corticosterone concentration. Further improvements and *in vitro* analysis should be conducted to better elucidate the underlying mechanism of the observed hypersensitivity, but also its resolution though exercise intervention. Western blot analysis of urogenital tissues, immunohistochemistry, pharmacological stabilization of mast cells, and gene expression analysis of urogenital tissues for neuroimmune profile will be invaluable in furthering these efforts.

Final conclusions

Idiopathic functional pelvic pain disorders, specifically IBS, CP/CPPS, and IC/PBS, are common, debilitating, and frequently co-diagnosed. A subset of patients report a history of abuse or neglect during early childhood, which is a critical period of nociceptive and stress-resilience programming, as well as a triggering or worsening of symptoms during periods of heightened stress. The mechanisms of chronic visceral pain are not well understood, in part due to its diffuse and poorly localized nature, often confused or overlapping between two visceral organs. Additionally, the diverse nature of visceral pain is compounded by multiple factors, including psychosocial stress, sexual dimorphism, and genetic and/or environmental predisposition. These multiple contributing factors make treatment and research efforts, especially the development and study of relevant animal models, challenging. However, visceral hypersensitivity has been recognized to occur due to (1) peripheral sensitization, (2) central sensitization, and (3) dysregulation of descending pathways that modulate spinal nociceptive

transmission [2]. Of note is the HPA axis; comorbid mood and pelvic pain disorders have been associated with disruption in proper functioning and limbic regulation of the HPA axis, which utilizes CRF to regulate stress response and influence the perception of pain [21, 101-105]. Furthermore, increased mast cell infiltration and/or activation has been implicated in IBS, CP/CPPS, and IC/PBS [34-36]. Further understanding of the development and maintenance of chronic pelvic pain will better inform appropriate assessment and interventions, thus leading to improved physical and psychological function. Due to the shared symptomology and comorbidity of functional pelvic pain disorders, it would be most advantageous to develop a reliable animal model depicting multiple conditions.

This work utilized a male mouse model of early life stress by neonatal maternal separation to conclude: 1) NMS in male mice disrupts inflammatory- and stress-induced gene expression in the colon, potentially contributing towards an exaggerated response to specific stressors later in life; 2) NMS in male mice induces perigenital hypersensitivity, enhances mast cell degranulation and inflammatory gene expression in urogenital organs, and results in decreased HPA axis output; furthermore, water avoidance stress enhanced mast cell degranulation and gene expression changes associated with stress sensitization, but did not exacerbate nociceptive behaviors; and 3) early and late exercise can prevent and reverse NMS-induced perigenital allodynia, as well as normalize mast cell degranulation in urogenital tissues.

Further improvements and *in vitro* analysis should be conducted to better elucidate the underlying mechanism of the observed hypersensitivity, but also its resolution though exercise intervention. For instance, BDNF can be more sensitively measured from serum using ELISA, while GTPγS binding assays can be used to evaluate ligand affinity for CRF_1 and/or CRF_2. Western blot analysis of urogenital tissues, immunohistochemistry, pharmacological stabilization of mast cells, and gene expression analysis of urogenital tissues for neuroimmune profile will be invaluable in furthering these efforts. Pharmacological inhibition of CRF_1 or genetic knock

out/down could also be performed to further investigate the relationship between NMS and HPA axis activity within this model in these contexts. Supplementary study of the impact of NMS on reward behavior and CNS signaling, specifically the dopaminergic reward pathway, is a particularly interesting thread of investigation.

We intended to identify key modulators that could serve as potential pharmacological targets, as well as provide the first pre-clinical evidence on the efficacy of an exercised-based therapeutic intervention, an easily translatable and inexpensive intervention. Positive results from this study could potentially be applied to other functional pain disorders linked to early life stress, including fibromyalgia and migraine, thus broadening the impact of our findings. The benefits/impact of the work would be improving the quality of life of individuals suffering from these debilitating mood and pain disorders, as well as decreasing the overall annual expense of treatment for these comorbid disorders.

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